JOURNAL OF EXPERIMENTAL BOTANY

AN OFFICIAL ORGAN OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY

Volume 12 *May* 1961 *Number* 35

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OXFORD: AT THE CLARENDON PRESS

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[*continued on p. iii of cover*

Changes in Content and Composition of the Fructose Polymers in Tubers of *Helianthus tuberosus* L. during Growth of Daughter Plants

T. G. JEFFORD AND J. EDELMAN

Department of Plant Physiology, Imperial College of Science & Technology, London, S.W.7.

WITH FIVE FIGURES IN THE TEXT

Received 6 October 1960

SUMMARY

Most of the dry weight of sprouting Jerusalem artichoke tubers was lost during the first few weeks of growth of the new shoots; at least 80 per cent. of this loss was accounted for by disappearance of polymerized fructose. It was demonstrated that much, if not all, of this material was translocated to the growing shoots, presumably in the form of sucrose. Depolymerization of fructose residues occurred during this process, and individual changes in the mono-, oligo-, and poly-saccharide fractions were consistent with the hypothesis that a specific hydrolytic enzyme, known to occur in tuber extracts, is involved.

INTRODUCTION

THE Jerusalem artichoke tuber contains a series of β-D-fructofuranosides, each member of which has the general formula G∼F-$(F)_n$ where G∼F denotes a sucrosyl group and n may be any number from zero (sucrose) to about 35 (inulin); the fructose residues are all 2:1[1] linked giving straight chains. Apart from cellulose, the tubers contain no other carbohydrates in significant amount. This series is widespread in the Compositae (Bacon and Edelman, 1951). Lower members of the series, e.g. the trisaccharide 1^F-fructosyl sucrose and the tetrasaccharide $(1^F\text{-fructosyl})_2$sucrose, have been found in other higher plants and are also produced by the action on sucrose of such transfructosylases as levansucrase and invertases from various sources (see reviews by Edelman, 1956, 1961; and Bacon, 1961).

Little is known of the significance of fructose polymers in higher plants, and it is even possible that they are merely by-products of carbon metabolism. As a preliminary to studying the enzymic relationships of these compounds, therefore, we have investigated their availability for further metabolism during active growth of daughter plants attached to artichoke tubers. This work forms part of a project to investigate the role of polymerized fructose in the metabolism of higher plants.

Although little is known of the biochemical fate of fructose polymers, some

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Although little is known of the biochemical fate of fructose polymers, some

work has been reported on changes in the series of fructosides of the artichoke tuber during overwinter storage. It has long been known that gradual decrease in the degree of polymerization (DP) occurs during this period, and that it is not possible to identify inulin in the juice after about December; this is accompanied by a change in sign of the optical rotation of the juice from negative to positive. Bacon and Loxley (1952) showed chromatographically that material of low DP (2 to about 6) accumulated at the expense of the more highly polymerized material, although the total sugar content remained constant. As each polymer chain contains a single glucose residue they inferred an increasing total glucose content produced presumably by isomerization of fructose residues. Although these authors continued their observations up to a time at which tubers gave rise to daughter plants, they did not examine the relationship between the changes in carbohydrate content and growth.

The results reported below demonstrate that most of the soluble carbohydrate of the tuber is lost during growth of the daughter plant and that much of it serves to provide substance to the growing tissue. During this time, detailed changes in the individual fructosides were followed and the results were consistent with the hypothesis that the hydrolytic β-D-fructofuranosidase described by Edelman and Bacon (1951) is involved in utilization of the polymerized material.

MATERIALS AND METHODS

Tubers were obtained from the Botanical Supply Unit, Englefield Green, Surrey; before use they were stored at 2° C. in damp sand.

Tubers were planted vertically in John Innes No. 1 Compost, unless otherwise stated, with their apices 1–2 in. below the surface. For determination of dry weight the tissue was sliced into tared metal trays and placed in an oven at 61° C. for 24–30 hr. The trays were allowed to cool to room temperature in a desiccator, weighed, and replaced in the oven; the treatment was repeated until constant weight was reached. Soluble carbohydrate was extracted by slicing the tissue into boiling 80 per cent. aqueous ethanol; boiling was continued for at least a further 5 min. It was assumed that this treatment killed tissue quickly enough to prevent the carbohydrates from being significantly altered by post-mortem enzymic action. The supernatant liquid was decanted and the slices covered with distilled water which was then brought to the boil and allowed to continue boiling vigorously for about 10 min. The supernatant was decanted. Two similar extractions by distilled water followed and the four extracts were combined. This procedure was considered to achieve practically quantitative extraction: a preliminary investigation showed that for every 1,000 mg. of combined fructose extracted in this manner, three subsequent treatments with boiling water extracted only 44, 14, and 8 mg. respectively. The bulked extract was exposed to a stream of air from a fan and evaporated to a volume between 10 and 40 ml. under an infra-red lamp or occasionally on a hot-plate. The pH of the extracts was always in the range of 6·5 to 5·8. The concentrated extracts were filtered through glass wool, made up to volume

in volumetric flasks with distilled water, and kept at 2° C. under toluene. Some extracts were evaporated to dryness in a vacuum desiccator over $CaCl_2$ and made up to volume just before use.

Descending chromatography was carried out on Whatman No. 1 paper. Normally between 2 and 3 mg. of total ketose was loaded as a streak over a distance of 10 cm. with a marker spot at either side. Chromatograms were developed for 42–48 hr. in *n*-propanol/ethyl acetate/water (7:1:2 v/v). After development the marker strips were cut off and sprayed with benzidine/trichloracetic acid (Bacon and Edelman, 1951). Compounds were eluted from the paper by distilled water after location by means of the markers. The eluates were evaporated to dryness over $CaCl_2$ in a vacuum desiccator, dissolved in a known volume of water, and samples removed for estimation of sugar.

Total fructose (free plus combined) was estimated using the method of Cole (adapted from Bacon and Bell, 1948). Reagents were measured using polythene 'rapid dispensers' ('E–MIL' Works, Nr. Pontypridd, Glam.); it was found that the polythene vessels did not interfere with either of the reagents and that measurement of volume was sufficiently accurate and consistent. Free glucose, after chromatographic separation, was estimated by the Somogyi (1952)–Nelson (1944) method. Sugar standards and reagent and paper blanks were included in every set of estimations.

RESULTS

Changes in weight and carbohydrate content of tubers during the growth of daughter plants. Tubers of similar size and shape were selected and divided into three groups of ten with fresh weights of 16–21 g., 22–24 g. and 27·5–36 g., respectively. The tubers were numbered and planted in random order in lots of six, each lot in a 10-in. earthenware pot, and placed in a heated greenhouse at 20±2° C. Samples were removed at intervals during a period of 7 weeks, one tuber being taken at random from each of the three groups on each occasion. Tubers were washed with tap water, dried with a towel, and the shoots and roots were cut off flush with the tuber surface. The shoots and tubers were then weighed. Roots were rejected, as it was not feasible to separate them quantitatively from the compost. Each tuber was cut in half longitudinally and the pieces weighed; one half was used for dry-weight determination and the other for extraction of soluble carbohydrate.

Although there was no apparent change in size of any of the tubers as judged qualitatively during growth of the daughter plants, slicing of the tubers revealed a progressive sponginess of the internal tissue. This was at first most apparent in the region a few millimetres beneath the epidermis. This sponginess became more marked as growth of the new plant continued and the affected region gradually spread towards the centre, while the outer layers of the tuber became harder and woody. After some 4–5 weeks there was a small firm central 'core' surrounded by a deep layer of drier stringy tissue

interspersed with many large gas-filled spaces, the whole contained in a rigid outer coating.

The average progressive decrease in dry weight and the corresponding loss of total fructose are shown in Fig. 1; curves for the individual groups were

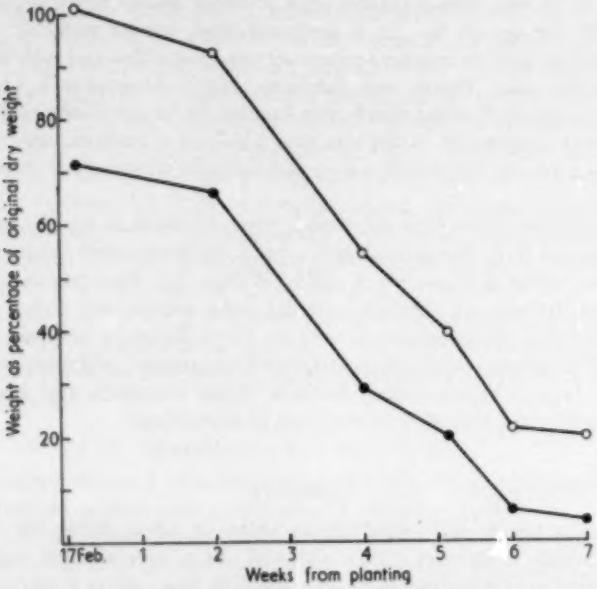

FIG. 1. Changes in dry weight ○, and anhydrofructose content ●, of tubers. Each value is an average of three observations.

closely similar to this average. Table I shows the overall decrease in dry weight compared to the loss of total fructose over the 7-week period for the three groups individually.

Similar relationships were found for two groups of tubers with fresh weights ranging from 56·5–69 g. and from 5·0–8·0 g., respectively; it is apparent that differences in tuber size had no significant effect on the pattern of change.

The tops grew rapidly during the experimental period, reaching a height of several feet by the 7th week, and it was obvious that photosynthesis would vitiate attempts to discover to what degree material lost from the tubers was translocated to the daughter plants. It was necessary in drawing up such a balance sheet, therefore, to prevent photosynthesis, and also to measure the dry weight of the roots (which were not recovered in this first experiment).

Tubers were therefore grown in continuous darkness at 20° C., singly in 6-in. pots containing fine washed sand to facilitate recovery of the roots. Samples were lifted at suitable intervals and the roots were carefully washed

TABLE I

Relationship between loss in dry weight and loss in total fructose from tubers during the growth of daughter plants

Changes for 7-week period; for details of the experiment see text.

Group (fresh weight g.)	Initial• fresh weight g.	Initial• dry weight g.	Loss in• dry weight g. (*a*)	Loss in•† total fructose g. (*b*)	(*b*) as % of (*a*)
16–21	19·5	3·96	3·29	2·73	83
22–24	22·3	5·14	4·15	3·49	84
27·5–36	31·8	7·17	5·47	4·73	86·5

• Calculated as the average for each group.
† Calculated as anhydro-fructose.

free of sand. Shoots and roots were cut off flush with the tuber surface, and fresh and dry weights determined.

Results shown in Fig. 2 show that changes in the dry weight of the tubers

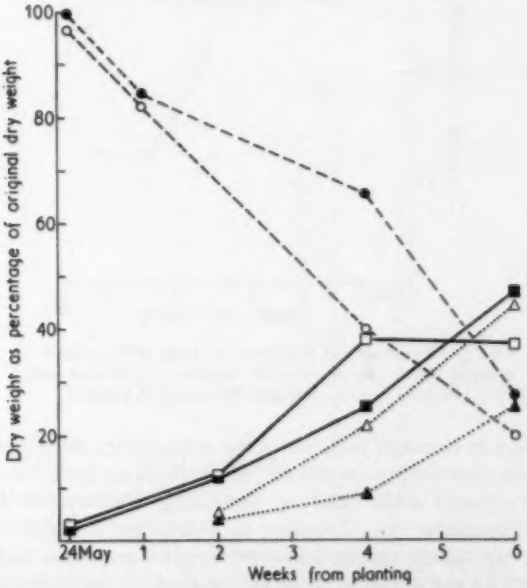

FIG. 2. Fate of material lost from tubers sprouted in darkness. ● Tuber. ■ Shoot plus root. ▲ Loss by respiration, calculated by difference. ○ □ △ Values for duplicate experiment.

were comparable with those shown in Fig. 1, and demonstrate that a substantial amount of material is in fact transported from the tuber to the growing plant. Respiration would account for that fraction of the dry weight lost entirely (Fig. 2 dotted line) but most of this was probably respired by the daughter

plant following translocation from the tuber. This can be deduced more clearly from results of an experiment in which the newly growing tissue was removed from tubers at a time when translocation from the latter was taking place rapidly. Twelve weighed tubers were planted in compost and allowed to grow in the open on a roof in South Kensington. When the daughter plants were about a foot high, at which stage carbohydrate was judged to be disappearing

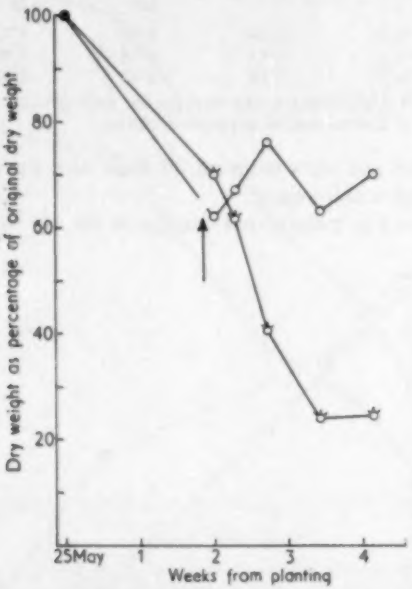

FIG. 3. Dry weight of tubers at intervals after removal of growing tissue. ● Original dry weight. ○ Growing tissue removed at time indicated by arrow. ⊗ Control.

from the tubers at maximal rate, six of the tubers were dug up and all roots, shoots, and dormant buds were cut off; the wounds on the tuber surface were immediately covered with vaseline containing Tetracycline–HCl (kindly donated by Cyanamid Ltd., London) to discourage invasion by micro-organisms, and the tubers replaced in their original positions in the pots. No decay occurred for the duration of the experiment. At intervals pairs of tubers of similar weight, one treated and one not, were sampled. Changes in dry weight are shown in Fig. 3 and are taken to indicate that no further loss of dry matter from the tuber occurs once the newly growing tissue is removed.

Changes in the individual carbohydrates. Table II gives results obtained for the tubers used in the experiment described on p. 179; the halves not used for dry weight determination (given in Fig. 1) were extracted for estimation of soluble carbohydrate.

TABLE II

Changes in individual carbohydrates present in tubers during the growth of daughter plants

For details of the experiment see text. Values are averages for 3 tubers, one from each fresh-weight group.

Period of growth (days)	Dry wt. mg./g. of original fresh wt.	Dry wt. mg./g. of current fresh wt.	Glucose mg./g. of original dry wt.	Fructose content, calculated as mg. free hexose/g. of original dry weight					
				Fructose	Sucrose	Tri-saccharide	Tetra-saccharide	Penta-saccharide	Poly-saccharide*
1	214	214	—	4·2	41·7	34·1	47·5	48·5	618
14	198	200	3·7	10·0	32·2	26·0	39·7	42·3	598
28	117	130	2·1†	1·0	36·3	14·2	10·1	9·4	142
36	84·0	103	1·2	74·5	32·4	13·4	9·0	9·2	82·6
42	46·0	70·0	1·1	39·0	12·3	4·2	2·7	2·5	7·6
49	43·0	110	3·0	17·3	9·2	2·7	2·1	2·7	11·0

* Total fructosides with DP $\geq$ 6.
† Average for two tubers.

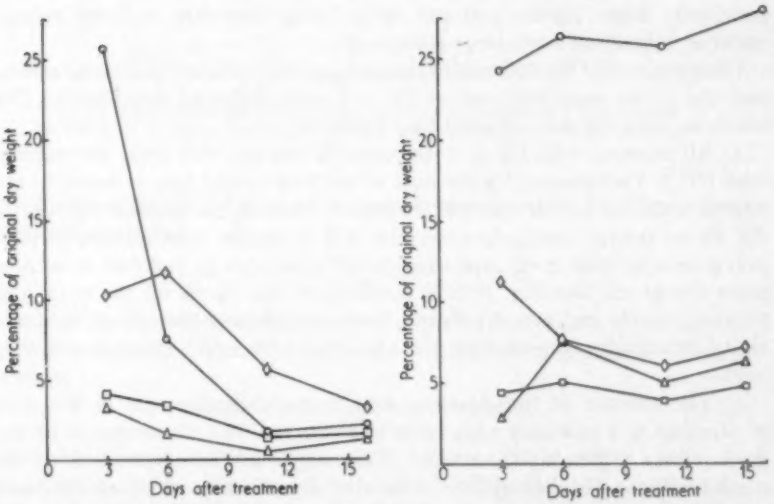

FIG. 4. Changes in the fructosides after removal of growing tissue from the tuber. Controls on left. Treated plants on right. ○ DP $\geq$ 5, △ DP 3 plus DP 4, □ DP 2 (sucrose), ◇ free fructose. All values are in terms of fructose content calculated as free hexose.

It should be noted that only the *fructose* content of the fructosides is given in this Table. Values for the glucose content of each of the compounds DP 2 to 5 can be readily calculated from the formula $G{\sim}F{-}(F)_n$, as n can be given known values; this cannot be done for the polysaccharide fraction.

The effect caused by the separation of the tissues of the daughter plant

from the tuber on these changes is shown in Fig. 4: these results were obtained by analysis of parts of the tubers used in the experiment described on p. 182 and should be compared with Fig. 3.

DISCUSSION

These results show that during the growth of the daughter plant considerable changes in the carbohydrate content of the parent tuber take place. Thus over a period of 7 weeks from planting some 80 per cent. of the dry weight of the tuber disappeared, and it was deduced from results of experiments performed in total darkness to prevent photosynthesis, and from experiments in which plants were removed from their parent tubers, that most, if not all, of this material was translocated to the growing plant, some of it being later respired. About 85 per cent. of this loss in dry weight was accounted for by a parallel disappearance of polymerized fructose. It is evident, therefore, that the fructosan of the tuber contributes markedly to the growth of the new plant. During this process the tubers changed very little in external appearance but when cut open gave every evidence of having been 'sucked out', the previously dense parenchymatous tissue being converted to loose stringy material containing many large gas spaces.

Changes in all of the detectable monosaccharides and short chain fructosides, and also in the total fructosan of DP $\geqslant 6$, were followed individually. The following facts became apparent (see Table II):

1. All fractions with DP $\geqslant 2$ decreased in amount with time, the material with DP $\geqslant 6$ accounting for the bulk of the total weight lost, as would be expected from the largest fraction. However, whereas the fructose content of the tetra-, penta-, and polysaccharides fell at similar rates relative to their initial content, that of the trisaccharide fell more slowly, and that of sucrose more slowly still (see Fig. 5). The similarity of the curves for the tetra- and pentasaccharide and mixed polymer fractions indicates that all of the individual fructosides ranging from DP 4 to DP n were losing fructose at similar rates.

2. The amount of free fructose rose from practically zero at the time of planting to a relatively high value at about the time of maximum breakdown of the polymerized material. This suggests direct hydrolysis of the combined fructose, although the amount of free fructose which accumulated at the highest level (110 mg./g. of the original dry weight) was very much less than the amount of depolymerization which had occurred by that time (642 mg.).

3. The level of free glucose remained low throughout, being always less than 4 mg./g. of the original dry weight.

These observations are consistent with the working hypothesis that the process of carbohydrate breakdown is initiated by the hydrolytic enzyme described by Edelman and Bacon (1951): we have assumed for this purpose that sucrose is the major translocatory substance (although the possibility of

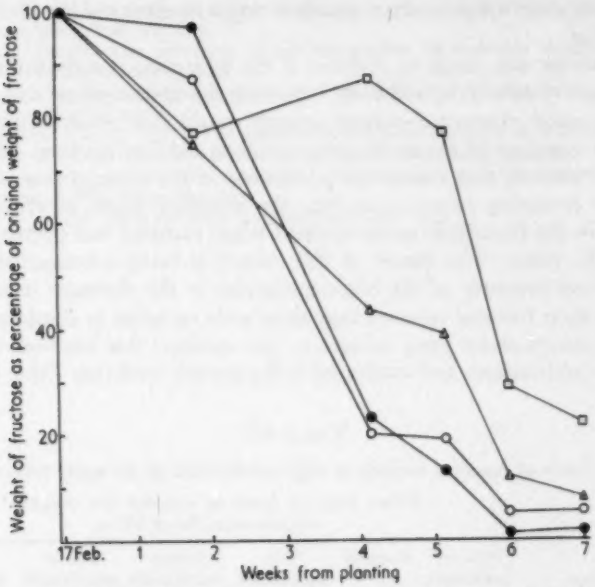

FIG. 5. Relative changes in fructose content of the fructosides of the tuber. ● DP ⩾ 6, ○ DP_5, ○ DP_4 (coincident with all values for DP_5), △ DP_3, □ DP_2 (sucrose).

direct translocation of polymerized material cannot be ruled out). This enzyme catalyses the reaction

$$G{\sim}F{-}(F)_n \rightarrow G{\sim}F{-}(F)_{n-1}{+}F$$

where the fructose is combined by 2:1[1] linkages and where $n \geqslant 1$; the sucrose link is not attacked. The last stage in the breakdown of a polymer chain is therefore,

$$\text{trisaccharide} \rightarrow \text{sucrose}+\text{fructose} \qquad (1)$$

and it may be expected that the fructose will be reconverted to sucrose by well-known reactions: thus, results to be published by Recaldin and Edelman have shown that fructose fed to tuber disks appears in the tissue in the form of sucrose.

The action of the hydrolase upon the fructosides of different DP would determine in two opposing ways the changes which would occur in the concentration of each individual member of the series: thus the amount of a member, DP x, will be decreased by direct attack, but increased by reduction in chain length of the next higher member, DP(x+1). As the lower fructosides did not, in fact, increase in concentration, it can be inferred that they are attacked more rapidly than the higher ones. Direct investigation of the specificity

of the hydrolase with respect to purified single members of the series is now in progress.

An attempt was made to discover if the hydrolase is indeed involved in the changes observed, by suddenly removing the carbohydrate sink, viz., the daughter plant; continued enzyme activity would then result in increases in the lower members of the series and/or sucrose and free fructose. The results showed, however, that a controlling influence in the changes observed in the normally sprouting tuber is, in fact, the daughter plant, as all detectable changes in the fructoside series stopped when material was prevented from leaving the tissue. The nature of this control is being investigated further.

A curious property of the oligosaccharides in the dormant tuber is that although their fructose contents may show wide variation in absolute amount, they are always about unity relative to one another; this has been shown in previous publications, and confirmed in the present work (see Table III). The

TABLE III

Ratio of fructose content in oligosaccharides of dormant tubers

Source	No. of estimates	Mean ratio of fructose content (by weight) to that of oligosaccharide of DP 5				
		Sucrose	Tri-saccharide	Tetra-saccharide	Penta-saccharide	Hexa-saccharide
Bacon and Edelman (1951)	10	1·16	0·90	1·11	(1·00)	1·06*
Bacon and Loxley (1952)	15	0·99	0·79	0·94	(1·00)	—
Present work	8	0·83	0·69	0·95	(1·00)	—
Total	33	1·05	0·80	0·99	(1·00)	1·06*

* Mean of 8 estimates.

similarity of these ratios implies an equilibration among the members of the series during dormancy. From the comments made above, it appears likely that, although sucrose and probably the trisaccharide do not conform during sprouting of the tuber, this equilibration continues during the breakdown of oligosaccharides of DP $\geqslant 4$ (see Table II and Fig. 5).

Such an equilibrium on the basis of fructose content *by weight*, rather than on the basis of the molarity of the individual oligosaccharides is difficult to understand. It implies that an enzyme system arranges the molecular population in such a way that the total number of fructose residues is the same for different members of the series. No explanation can as yet be given for this phenomenon.

LITERATURE CITED

BACON, J. S. D. (1961). The Oligofructosides. *Bull. Soc. chim. biol.* (in press).

—— and BELL, D. J. (1948). Fructose and glucose in the blood of the foetal sheep. *Biochem. J.* **42**, 397.

—— and EDELMAN, J. (1951). The carbohydrates of the Jerusalem artichoke and other Compositae. Ibid. **48**, 114.

BACON, J. S. D. and LOXLEY, R. (1952). Seasonal changes in the carbohydrates of the Jerusalem artichoke tuber. Ibid. **51**, 208.

EDELMAN, J. (1956). The formation of oligosaccharides by enzymic transglycosylation. *Advances in Enzymology* **17**, 189.

—— (1961). Transfructosylation in plants and especially in *Helianthus tuberosus* L. *Bull. Soc. chim. biol.* (in press).

—— and BACON, J. S. D. (1951). The action of a hydrolytic enzyme system from *Helianthus tuberosus* L. on carbohydrates present in the tubers. *Biochem. J.* **49**, 446.

NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **153**, 375.

SOMOGYI, M. (1952). Notes on sugar determination. Ibid. **195**, 19.

Some Characteristics and Inhibitors of Indoleacetic Acid Oxidase from Tissue Cultures of Crown-gall

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WITH FOUR FIGURES IN THE TEXT

Received 19 November 1960

SUMMARY

Extracts from tissue cultures of crown-gall from *Parthenocissus* catalysed the destruction of indoleacetic acid *in vitro* with optimum activity at pH 4·5. The presence of two co-factors, Mn^{++} and 2,4-dichlorophenol, was necessary for this activity, which was found to be strictly aerobic.

Chlorogenic acid, caffeic acid, scopoletin, ferulic acid, and gibberellic acid markedly inhibited IAA-destruction. Chlorogenic acid inhibition was reversed by the addition of H_2O_2. Chlorogenic acid was not oxidized by the IAA-destroying system and did not behave as a competitive inhibitor.

The IAA-oxidase extract manifested peroxidase and phenolase activity with catechol and pyrogallol as substrates. However, this activity was greater at pH 7·0 than at the optimum for IAA-oxidase activity, pH 4·5. Further evidence of the existence of these two enzymes in the intact tissue was demonstrated by histochemical studies. In tissue slices, peroxidase activity was very high and widely distributed while phenolase activity was low and restricted to localized centres of the tissue.

INTRODUCTION

ENZYMES capable of oxidizing indole-3-acetic acid (IAA) occur in a number of higher plants, ferns, and fungi (Gordon, 1954; Larsen, 1951; Ray, 1958) although the mechanism of action for these enzymes is not completely known. IAA-oxidases from various plant sources differ in pH optimum and in the requirement of a phenolic compound for activity. Extracts from wheat leaves (Waygood *et al.*, 1956*b*), pineapple (Gortner and Kent, 1953), *Lupinus albus* (Stutz, 1957), *Polyporus versicolor* (Tonhazy and Pelczar, 1954), *Vicia faba* and *Helianthus annuus* (Thimann, 1934), quackgrass (*Agropyron repens*) rhizomes (Mudd *et al.*, 1959), and etiolated pea epicotyls (Tang and Bonner, 1947) require manganese (Mn^{++}) for complete or enhanced activity. However, not all IAA-oxidase systems require the incorporation of a phenolic factor. Extracts from pineapple (Gortner and Kent, 1953) and *Omphalia flavida* (Ray and Thimann, 1956) show optimum IAA-oxidase at pH 3·5, whereas the IAA-oxidase from etiolated pea epicotyls (Tang and Bonner, 1947) and bean roots (Wagenknecht and Burris, 1950) exhibit optimum activity in the range of pH 6–7. The IAA-oxidases of wheat leaves (Waygood *et al.*, 1956*b*) and *Polyporus versicolor* (Tonhazy and Pelczar, 1954) show pH optima at 5·0–6·0 and 4·5, respectively.

From the above evidence, it seems possible that the IAA-oxidizing systems from different plant sources employ different mechanisms of action. On the other hand, similarities in oxygen requirement, stoichiometry of the reactions, peroxidase activity (Ray, 1958), and inhibition by polyphenols (Gordon, 1954; Gortner and Kent, 1953; Rabin and Klein, 1957; Ray, 1958) suggest strongly that the same or very similar enzyme systems are responsible for IAA destruction.

Crown-gall from *Parthenocissus tricuspidata* (Sieb. and Zucc.) Planch. releases into the external medium one or more enzymes which are capable of peroxidative activity and, in the presence of 2,4-dichlorophenol (DCP) and Mn^{++}, IAA destruction.

This investigation deals with a partial characterization of the IAA-oxidase present in extracts from crown-gall of *Parthenocissus* and the effects on that system caused by the separate incorporation of certain putative inhibitors. These results should illustrate the similarities or differences of the crown-gall enzyme as compared or contrasted to other IAA-oxidases previously studied.

MATERIALS AND METHODS

Crown-gall tissue from *Parthenocissus* was cultured on White's medium (White, 1943) for a period of 4 to 5 weeks prior to its utilization as a source of IAA-oxidase. Cell-free extracts of the tissue were prepared in the following manner: crown-gall tissue was sliced into thin sections (1–2 mm.), suspended in an equal weight of 0·02 M. KH_2PO_4–0·02 M. potassium acid phthalate buffer and allowed to stand for 1 hour. The extract was strained through a double layer of cheesecloth and the cellular debris was discarded. The remaining liquid was centrifuged at 700 *g* for 20 minutes. After centrifugation, the resultant supernatant was decanted and used as a source of IAA-oxidase. All the above operations were carried out at 0–4° C. Extracts were capable of oxidizing IAA for as long as 4 days after preparation if kept frozen.

IAA-oxidase reactions were run at room temperature in 25-ml. Erlenmeyer flasks containing the following reaction mixture: DCP and $MnCl_2$, both in concentrations of 10^{-4} M. (Lipetz and Galston, 1959), IAA (200 μgm.), enzyme (1 ml.), and 0·02 M. KH_2PO_4–0·02 M. $KHC_8H_4O_4$ buffer in a final volume of 10 ml. The reaction was started by the addition of the enzyme; inactivated enzyme was added to controls. For complete inactivation, enzyme preparations were maintained at 100° C. for one half-hour. At the end of the reaction time, a modification of the procedure of Gordon and Weber (1951) for determining residual IAA was followed. 4-ml. aliquots of the reaction mixtures were added to 8 ml. of Salkowski reagent (1 ml. 0·5 M. $FeCl_3$ in 50 ml. 35 per cent. $HClO_4$). After 30 minutes, residual IAA was determined colorimetrically in a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter. Platt and Thimann (1956) have indicated that this assay method is subject to interference from various sources. In view of their findings extreme care was taken to avoid the use of interfering substances in

our experiments. The enzyme extract gave no colour with Salkowski reagent nor did it interfere with the quantitative determination of IAA by this method. All chemical reagents used in our experiments were tested for their possible interference in the assay procedure, and from our results we concluded that this procedure was satisfactory for the purposes of this research.

Studies with purified horse-radish peroxidase (Worthington Biochemical Corporation) were carried out essentially as described by Galston *et al.* (1953). Reaction mixtures containing H_2O_2 (0·0003 per cent.), IAA (200 μgm.), horse-radish peroxidase (60 μgm.), and 0·05 M. sodium citrate–0·1 M. dibasic sodium phosphate buffer (pH 5·6) in a final volume of 10 ml. were incubated for 40 minutes at 25° C. Residual IAA was determined as described above.

Chlorogenic acid was quantitatively determined spectrophotometrically according to the method of Shiroya *et al.* (1955).

For histochemical studies on crown-gall tissue slices, one or two drops of gum guaiacum solution (1 per cent. in 70 per cent. ethanol) were added and followed by two drops of 0·003 per cent. H_2O_2, the latter being omitted from the control.

RESULTS

The necessity of Mn^{++} and DCP for the destruction of IAA by the crown-gall enzyme system as reported by Lipetz and Galston (1959) was confirmed in this investigation. In addition, it was found that the destruction of IAA by this system did not occur in the absence of oxygen. The results of several experiments clearly indicated that optimum enzyme activity occurred at pH 4·5 (Fig. 1). A time-course study of the destruction of IAA by the crown-gall enzyme was conducted. After an initial lag period, the rate of IAA destruction was linear over a 30–40 minute interval (Fig. 2).

Preliminary experiments run at pH 6·1 resulted in IAA-oxidase inhibition by minute concentrations of chlorogenic acid. At this pH approximately 2·5 μgm. of chlorogenic acid were necessary for 50 per cent. inhibition. However, at pH 4·5 (Fig. 3), chlorogenic acid inhibition was more pronounced, since only 0·08 μgm. were sufficient to accomplish the same degree of inhibition. It is also apparent that the degree of inhibition is a function of the concentration of the inhibitor. In order to determine whether chlorogenic acid was a competitive inhibitor of the IAA-oxidase, an enzyme preparation was divided into two portions. One portion of the enzyme preparation was used for linear rate reaction determinations, and the other was used for experiments from which Lineweaver–Burk plots (Lineweaver and Burk, 1934) were prepared. Lineweaver–Burk plots of the rate of IAA destruction in the absence of chlorogenic acid were linear and intercepted at the vertical axis. However, it could not be positively determined at pH 6·1 or 4·5 whether chlorogenic acid inhibition was of a competitive nature. When the results of such experiments were plotted, the reaction mixtures containing chlorogenic acid repeatedly failed to follow normal enzyme kinetics. The lines, although straight, did not intercept at the vertical axis.

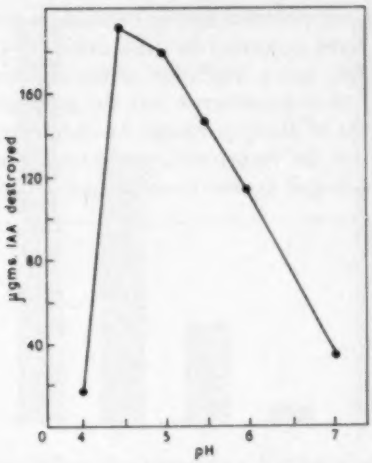

FIG. 1. IAA oxidase activity at several hydrogen-ion concentrations. Reaction mixtures contained the following: 2,4-DCP (10^{-4} M.), $MnCl_2$ (10^{-4} M.), IAA (200 µgm.), enzyme (1 ml.), and sufficient buffer to bring the final volume to 10 ml. Incubation time, 1 hr.; temp. 25° C.

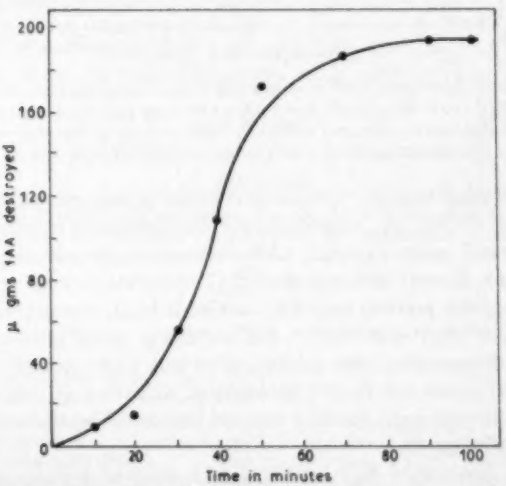

FIG. 2. Time-course study of the destruction of IAA by the crown-gall IAA-oxidase. Each reaction mixture contained: 2,4-DCP (10^{-4} M.), $MnCl_2$ (10^{-4} M.), enzyme (1 ml.), IAA (200 µgm.), and sufficient buffer to bring the final volume to 10 ml. Temp. 25° C., pH 4·5.

The destruction of IAA by horse-radish peroxidase in the presence of H_2O_2 was *not* inhibited by the addition of several concentrations of chlorogenic acid, nor was this oxidation inhibited when the amount of chlorogenic acid was constant with varying concentrations of added enzyme. Since

chlorogenic acid did not inhibit a known peroxidase system, and in view of the possible mechanisms proposed for IAA-oxidases (Galston *et al.*, 1953; Gordon, 1954; Larsen, 1951), inhibition of the crown-gall enzyme by this compound could be due to interference with the generation and/or utilization of the small quantities of H_2O_2 produced by the system. Consequently, it was expected that, by the incorporation of small quantities of peroxide, inhibition of the crown-gall system by chlorogenic acid might be reversed.

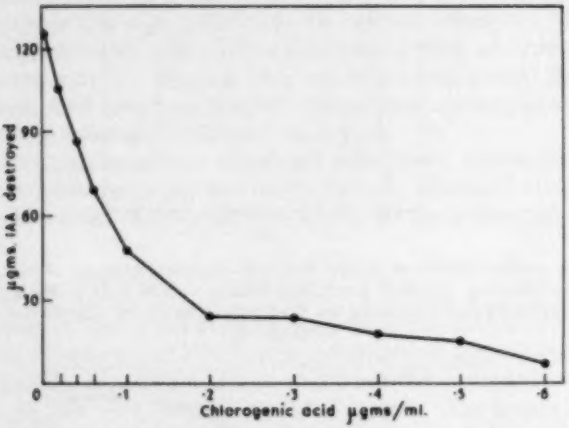

FIG. 3. The effect of chlorogenic acid on crown-gall IAA-oxidase activity. Reaction mixtures contained: 2,4-DCP (10^{-4} M.), $MnCl_2$ (10^{-4} M.), IAA (200 µgm.), enzyme (1 ml.), varying concentrations of chlorogenic acid, and sufficient buffer to bring to the final volume of 10 ml. Incubation time, 1 hr.; temp. 25° C.; pH 4·5.

Complete reversal of inhibition in flasks containing enzyme, chlorogenic acid, and H_2O_2 resulted (Fig. 4). Although controls containing inactivated enzyme and H_2O_2 showed some apparent IAA destruction, presumably due to the presence of H_2O_2 alone (Galston *et al.*, 1953), complete reversal appeared to be due to the enzymatic activity for which available H_2O_2 is required. Increased concentrations of DCP and Mn^{++} did not bring about inhibition reversal. It was also demonstrated that inhibition of the IAA-oxidase system from crown-gall tissues was not due to preferential oxidation of chlorogenic acid. Chlorogenic acid (200 µgm./10 ml.) was not destroyed by the enzyme system capable of destroying IAA.

Caffeic acid ($5{\cdot}6 \times 10^{-6}$ M.) inhibited IAA-oxidase activity as effectively as did chlorogenic acid. However, the other moiety of chlorogenic acid, quinic acid ($5{\cdot}2 \times 10^{-6}$ M.), had no inhibitory effect on the system. Scopoletin ($3{\cdot}6 \times 10^{-4}$ M.) completely inhibited the destruction of 200 µgm. of IAA at pH 4·5, gibberellic acid (10^{-5} M.) was responsible for approximately 50 per cent. inhibition, and ferulic acid (5×10^{-6} M.) inhibited IAA destruction by about 76 per cent. (Table I).

Lipetz and Galston (1959) showed that a peroxidase was released from

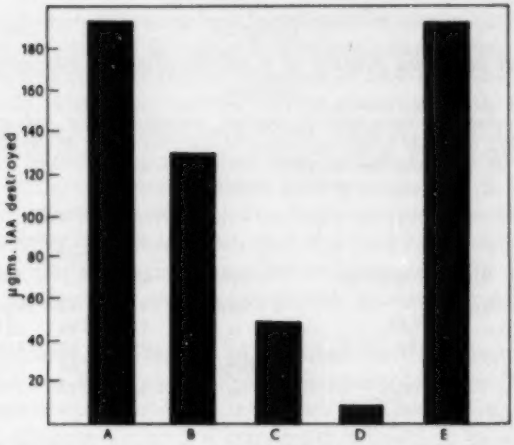

FIG. 4. The effect of added H_2O_2 on the chlorogenic acid inhibition of crown-gall IAA-oxidase activity. Reaction mixtures A–E indicated in the figure contained the following substances in the final concentrations indicated:

A. Enzyme, 1 ml.; 2,4-DCP, 10^{-4} M.; $MnCl_2$, 10^{-4} M.; IAA 200 µgm., H_2O_2, 0·0003%.
B. Same as A but lacking H_2O_2.
C. Same as A but enzyme inactivated plus chlorogenic acid, 6 µgm.
D. Same as A plus chlorogenic acid, 6 µgm.
E. Same as A plus chlorogenic acid, 6 µgm. plus H_2O_2, 0·0003%.

Final volume, 10 ml.; incubation time, 40 min.; temp. 25° C.; pH 4·5.

TABLE I

Inhibition of crown-gall IAA-oxidase activity by some naturally occurring plant compounds

Reaction mixtures contained the following: 2,4-DCP (10^{-4} M.), $MnCl_2$ (10^{-4} M.), IAA (200 µgm.), enzyme (1 ml.), added compounds in the concentrations indicated and sufficient buffer to make a final volume of 10 ml. Incubation time, 1 hr.; temp. 25° C.; pH 4·5

Inhibitor	Final conc. (M.)	µgm. IAA destroyed	% inhibition
None	0	192	0
Chlorogenic acid	$2·8 \times 10^{-6}$	0	100
Caffeic acid	$5·5 \times 10^{-6}$	0	100
Quinic acid	$5·2 \times 10^{-6}$	192	0
Scopoletin	$3·6 \times 10^{-4}$	0	100
Gibberellic acid	10^{-3}	102	46·9
Ferulic acid	$5·0 \times 10^{-6}$	46	76

crown-gall tissue slices into the external medium. Consequently, standard peroxidase determinations were run in order to test the extract for such activity. Peroxidase, as well as polyphenol oxidase, activity was evidenced when either pyrogallol or catechol was substituted for IAA and the reaction mixtures run at several hydrogen-ion concentrations (Table II). It should be noted that peroxidase and phenolase activities were greater at pH 7·0 than

TABLE II

Peroxidase and phenolase activity of a cell-free extract from crown-gall tissue cultures

The following substances were used in reaction mixtures where indicated: 2,4-DCP (10^{-4} M.), $MnCl_2$ (10^{-4} M.), H_2O_2 (0·0003%), catechol 0·5%, pyrogallol (0·5%), and sufficient buffer to bring reaction mixtures to a final volume of 10 ml. Incubation time, 40 min.; temp. 25° C.

Reaction mixture	Substrate	Klett units (pH) 4·5	6·0	7·0
Enzyme . . .	Pyrogallol	23	33	230
,, $+H_2O_2$.	,,	72	144	331
,, +Mn, DCP .	,,	21	54	250
,, . . .	Catechol	5	28	111
,, $+H_2O_2$.	,,	56	110	126
,, +Mn, DCP .	,,	11	110	163

at the lower hydrogen-ion concentrations, at which IAA destruction was greatest (Fig. 1).

Histochemical studies gave evidence that a high concentration of peroxidase is present in crown-gall tissue. There was also evidence of very localized phenolase activity.

DISCUSSION

Crown-gall tissue cultures from *Parthenocissus* produce an enzyme which is released into the external medium and is capable of destroying IAA *in vitro*. The release of an IAA-oxidase into the external medium by *Polyporus versicolor* (Tonhazy and Pelczar, 1954), etiolated pea stem tissue (Hillman and Galston, 1957), and *Omphalia flavida* (Sequeira and Steeves, 1954) has also been reported.

An absolute requirement of DCP and Mn^{++} for the crown-gall IAA-oxidase was initially demonstrated by Lipetz and Galston (1959), and their findings were confirmed in this study. The exact role of these co-factors in this system, or other previously studied IAA-oxidases, has not been completely elucidated (Gortner and Kent, 1953; Stutz, 1957; Waygood *et al.*, 1956*a*).

Preliminary investigations with the crown-gall enzyme were conducted at pH 6·1, since this pH was used by earlier investigators (Lipetz and Galston, 1959). However, the optimum pH of 4·5 for the IAA-oxidase extract, as determined by experiments reported here, agrees with the pH optimum reported for the IAA-destroying enzyme produced by *Polyporus versicolor* (Tonhazy and Pelczar, 1954). The pH optimum for these two systems is apparently different from the optima of other IAA-oxidase systems which range from pH 3·5 to 7·0 (Rabin and Klein, 1957; Ray, 1958; Ray and Thimann, 1956; Tonhazy and Pelczar, 1954; Wagenknecht and Burris, 1950). Apparent differences in pH optima do not necessarily negate the possibility that IAA-oxidases from different plant sources are similar in their mode of

action. The reported differences in pH optimum for certain systems may have been obtained as a result of the use of buffers composed of citrate which have been demonstrated to lower the pH optimum of IAA-oxidases (Mudd *et al.*, 1959). Similarly, it has been reported that the optimum pH for IAA destruction in dialysed pea homogenates was shifted from pH 5·0, in the absence of Mn^{++}, to pH 4·0 in the presence of Mn^{++} (Ray, 1958). The incorporation of Mn^{++} may be responsible for the inconsistencies in reported pH optima for some IAA-oxidases when a co-factor was used to enhance activity. It was not possible to determine whether this shift in pH optimum would occur in crown-gall IAA-oxidase reaction mixtures, as Mn^{++} and 2,4-DCP are absolute requirements and do not merely increase the activity of this system.

Naturally occurring inhibitors of IAA-oxidases have been reported in pineapple (Gortner and Kent, 1953), *Agropyron repens* rhizomes (Mudd *et al.*, 1959), callus tissue from *Parthenocissus* (Lipetz and Galston, 1959), and many other plants. Chlorogenic acid, a compound believed to be similar to the naturally occurring inhibitor in pineapple, markedly inhibited the pineapple oxidase (Gortner and Kent, 1953). Although the inhibitory action and absorption spectrum of chlorogenic acid and the naturally occurring inhibitor are similar, Gortner and Kent (1953) suggest that these compounds are distinct. Although there was no indication that chlorogenic acid acted as a competitive inhibitor in the pineapple system, Rabin and Klein (1957) reported that chlorogenic acid competitively inhibited pea IAA-oxidase.

When chlorogenic acid was incorporated into crown-gall IAA-oxidase reaction mixtures, greater inhibition resulted at pH 4·5 than at pH 6·1. Unlike the enzyme from etiolated pea epicotyls (Rabin and Klein, 1957), the crown-gall system did not appear to be inhibited competitively. Since the crown-gall extract contains at least two different enzymes, peroxidase and phenolase, it is possible that normal enzyme kinetics could not be demonstrated owing to an interaction between the enzymes present and one or more components of the extract with chlorogenic acid. However, there is no conclusive evidence that the chlorogenic acid is not a competitive inhibitor of the enzyme responsible for IAA destruction. Since Lineweaver–Burk plot preparations are used to demonstrate competition between inhibitor and substrate and to give an evaluation of the affinity of the substrate for the enzyme (Mehler, 1957), it would appear that this technique may not be suitable for studying relatively crude enzyme preparations.

A mechanism for IAA destruction involving the production of free radicals which may react with oxygen in the presence of Mn^{++} to form H_2O_2 has been suggested (Yamazaki and Souzu, 1960). In consideration of the absolute requirements for Mn^{++} and DCP the same or similar mechanism involving the production of free radicals and/or H_2O_2 may be effective for the crown-gall IAA-oxidase. If this is true, there are many possible causes of the inhibitory effect of chlorogenic acid that may alter expected results from Lineweaver–Burk plot preparations. The reversal of chlorogenic acid inhibition by the

addition of small quantities of H_2O_2 would seem to indicate that the synthesis or utilization of H_2O_2 or a suitable hydrogen acceptor is inhibited by chlorogenic acid. However, it does not seem likely that chlorogenic acid is interfering with the promoting effects of Mn^{++} or DCP, as increased concentrations of these two necessary co-factors in the presence of chlorogenic acid did not bring about reversal of inhibition. This phase of chlorogenic acid inhibition is worthy of future investigation in view of the possible interference with free radicals by chlorogenic acid. The production of such radicals in the course of IAA destruction has been suggested (Maclachlan and Waygood, 1956; Ray and Thimann, 1956; Waygood *et al.*, 1956*a*; Yamazaki and Souzu, 1960).

In view of the reversal of chlorogenic acid inhibition by peroxide, the mechanism for IAA-oxidase proposed by Galston *et al.* (1953), whereby a light-activated flavoprotein produced H_2O_2 and a peroxidase utilizes this H_2O_2 in the oxidation of IAA, cannot be ignored. However, unlike many other polyphenols that inhibit IAA-oxidases (Ray, 1958), chlorogenic acid does not appear to be competing for the H_2O_2 for its own oxidation, as the crown-gall IAA-oxidase system does not oxidize chlorogenic acid in the absence or presence of IAA. If chlorogenic acid were competing in this manner for the enzyme site, it does not seem likely that the small amounts used in reaction mixtures would completely inhibit the destruction of 200 μgm. of IAA. The reversal of inhibition by H_2O_2 strongly suggests that the production or utilization of H_2O_2 is being inhibited, although chlorogenic acid probably is not interacting directly with the H_2O_2 produced. The exact type of inhibition cannot be determined until the sequence of events that takes place in IAA destruction by the crown-gall extract has been clarified.

The separate incorporation of certain putative inhibitors (Table I) into crown-gall IAA-oxidase reaction mixtures was attempted in order to determine if the same effects of these substances would be consistent with the reported findings for other IAA-oxidase systems. The crown-gall and etiolated pea enzymes are similar, in that caffeic acid is the active inhibitory moiety of chlorogenic acid, while quinic acid has no inhibitory effect. Scopoletin, naturally present in a number of plants (Andreae and Andreae, 1949; Goodwin and Kavanagh, 1949), and reported to inhibit other IAA-oxidase systems (Andreae, 1952; Gortner and Kent, 1953), acted similarly on the crown-gall enzyme, but was not an inhibitor of the pea oxidase (Rabin and Klein, 1957) at the pH optimum for activity of that system.

Although approximately 50 per cent. inhibition of the system resulted when gibberellic acid was incorporated into reaction mixtures, it is possible that this is only an apparent inhibition and not a true indication of this substance's capabilities as an inhibitor. As indicated by Henderson (1960), the stability of this substance is questionable in even a weakly acid medium.

A number of workers agree that the enzyme responsible for the destruction of IAA *in vivo* is a special type of peroxidase (Galston *et al.*, 1953; Lipetz and Galston, 1959; Stutz, 1957; Yamazaki and Souzu, 1960). It has been

suggested that phenolases may participate in the destruction of IAA (Briggs and Ray, 1956; Thimann, 1934; Wagenknecht and Burris, 1950). As a result of the present study (Table II), a question arises as to the role of phenolase in the destruction of IAA by the crown-gall tissue. Peroxidase is perhaps primarily responsible for auxin destruction in the intact tissue, as histochemical studies indicate that phenolase activity is very low and limited to localized centres. It should be noted that the activity of the two enzymes in the crown-gall extract, as determined by catechol and pyrogallol oxidation, appears to be extremely low at the hydrogen-ion concentration for optimum activity of the IAA-oxidase. Although one or both of these enzymes may be involved in IAA destruction, the exact mechanism whereby one enzyme oxidizes pyrogallol and catechol at pH 7·0 as opposed to IAA oxidation at pH 4·5 cannot be explained at this time.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. W. A. Andreae for providing the scopoletin, to Dr. E. L. Davis for the gibberellic acid, and to Dr. R. M. Klein for the chlorogenic acid and other inhibitors used in this study. We are equally indebted to Dr. D. S. Van Fleet for his assistance in the histochemical aspects of this research and to Mrs. Eugenie Stacey for her able technical assistance. This research was supported in part by a grant (C-4052) to the second author from the Public Health Service, National Cancer Institute.

LITERATURE CITED

Andreae, S. R., and Andreae, W. A. (1949). The metabolism of scopoletin by healthy and virus infected tubers. *Can. J. Res.* **27,** 15.

Andreae, W. A. (1952). Effect of scopoletin on indoleacetic acid metabolism. *Nature,* **170,** 83.

Briggs, W. R., and Ray, P. M. (1956). An auxin inactivating system involving tyrosinase. *Plant Physiol.* **31,** 165.

Galston, A. W., Bonner, J., and Baker, R. J. (1953). Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. *Arch. Biochem. Biophys.* **42,** 456.

Goodwin, R. H., and Kavanagh, F. (1949). The isolation of scopoletin, a blue-fluorescing compound from oat roots. *Bull Torrey Bot. Club,* **76,** 255.

Gordon, S. A. (1954). Occurrence, formation, and inactivation of auxin. *Ann. Rev. Plant Physiol.* **5,** 341.

—— and Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. *Plant Physiol.* **26,** 192.

Gortner, W. A., and Kent, M. (1953). Indoleacetic acid oxidase and an inhibitor in pineapple tissue. *J. Biol. Chem.* **204,** 593.

Henderson, J. (1960). Influence of hydrogen ion concentration and autoclaving on gibberellin. *Nature,* **185,** 628.

Hillman, W. S., and Galston, A. W. (1957). Inductive control of indoleacetic acid oxidase activity by red and near infrared light. *Plant Physiol.* **32,** 129.

Larsen, P. (1951). Formation, occurrence, and inactivation of growth substances. *Ann. Rev. Plant. Physiol.* **2,** 169.

Lineweaver, H., and Burk, D. (1934). Determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* **56,** 658.

Lipetz, J., and Galston, A. W. (1959). Indoleacetic acid oxidase and peroxidase in normal and crown-gall tissue cultures of *Parthenocissus tricuspidata. Amer. J. Bot.* **46,** 193.

Maclachlan, G. A., and Waygood, E. R. (1956). Kinetics of the enzymatically catalyzed oxidation of indoleacetic acid. *Can. J. Biochem. Physiol.* **34,** 1233.

Mehler, A. H. (1957). *Introduction to Enzymology.* Academic Press Inc., New York.

MUDD, J. B., JOHNSON, B. G., BURRIS, R. H., and BUCHHOLTZ, K. P. (1959). Oxidation of indoleacetic acid by quackgrass rhizomes. *Plant Physiol.* **34**, 144.

PLATT, Jr., R. S., and THIMANN, K. V. (1956). Interference in Salkowski assay of indoleacetic acid. *Science*, **123**, 105.

RABIN, R. S., and KLEIN, R. M. (1957). Chlorogenic acid as a competitive inhibitor of indoleacetic acid oxidase. *Arch. Biochem. Biophys.* **70**, 11.

RAY, P. M. (1958). Destruction of auxin. *Ann. Rev. Plant. Physiol.* **9**, 81.

—— and THIMANN, K. V. (1956). The destruction of indoleacetic acid I. Action of an enzyme from *Omphalia flavida*. *Arch. Biochem. Biophys.* **64**, 175.

SEQUEIRA, L., and STEEVES, T. A. (1954). Auxin inactivation and its relation to leaf drop caused by the fungus *Omphalia flavida*. *Plant Physiol.* **29**, 11.

SHIBOYA, M., SHIBOYA, T., and HATTORI, S. (1955). Studies on the browning and blackening of plant tissues IV. Chlorogenic acid in the leaves of *Nicotiana tabacum*. *Physiol. Plant.* **8**, 594.

STUTZ, R. E. (1957). The indole-3-acetic acid oxidase of *Lupinus albus* L. *Plant Physiol.* **32**, 31.

TANG, Y. W., and BONNER, J. (1947). The enzymatic inactivation of indoleacetic acid I. Some characteristics of the enzyme contained in pea seedlings. *Arch. Biochem.* **13**, 11.

THIMANN, K. V. (1934). Studies on the growth hormones of plants VI. The distribution of growth substances in plant tissues. *J. Gen. Physiol.* **18**, 23.

TONHAZY, N. E., and PELCZAR, M. J. (1954). Oxidation of indoleacetic acid by an extracellular enzyme from *Polyporus versicolor* and a similar oxidation catalyzed by nitrite. *Science*, **120**, 141.

WAGENKNECHT, A. C., and BURRIS, R. H. (1950). Indoleacetic acid inactivating enzymes from bean roots and pea seedlings. *Arch. Biochem.* **25**, 30.

WAYGOOD, E. R., OAKS, A., and MACLACHLAN, G. A. (1956*a*). The enzymatically catalyzed oxidation of indoleacetic acid. *Amer. J. Bot.* **34**, 905.

—— —— —— (1956*b*). On the mechanism of indoleacetic acid oxidation by wheat leaf enzymes. *Can. J. Bot.* **34**, 54.

WHITE, P. R. (1943). *A Handbook of Plant Tissue Culture*. Jacques Cattell Press, Lancaster, Pennsylvania.

YAMAZAKI, I., and SOUZU, H. (1960). The mechanism of indoleacetic acid oxidase reaction catalyzed by turnip peroxidase. *Arch. Biochem. Biophys.* **86**, 294.

Evidence for a Factor which Prevents the Development of Parthenocarpic Fruits in the Strawberry

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WITH SIX FIGURES IN THE TEXT

Received 19 September 1960

SUMMARY

Observations of fruit development in the cultivated strawberry (*Fragaria* × *Ananassa* Duchesne) are interpreted as showing that, in unpollinated fruits, development of fruit tissues ceases at anthesis because of an inhibitory factor produced by the maturing carpels. It is suggested that one of the functions of pollination, or fertilization, is to remove this inhibition and make the tissues susceptible to stimulation by growth substances produced either by the parent plant or the fertilized carpels.

Receptacles in which pollination is confined to a single carpel swell only around the point of insertion of that carpel, and no swelling occurs beneath neighbouring unfertilized carpels. Likewise, after treatment of the plant with gibberellic acid swelling of fruit tissue occurred only at the base and did not extend into areas occupied by unfertilized carpels.

Parthenocarpic fruits were produced in two ways; both depended on the suppression of carpel development on the receptacle. In one case, modification of the plants' environment resulted in the production of leafy bracts in place of carpels. In the other, carpel development was specifically inhibited by applications of maleic hydrazide at the time of carpel formation.

When carpels were only partially suppressed swelling occurred only in areas either free of carpels or occupied by fertilized carpels, never in areas occupied by unfertilized carpels.

INTRODUCTION

IT is characteristic of the development of practically all fruits that after the ovaries mature at anthesis further development of tissues which form the fruit ceases and is not resumed until after pollination and the successful fertilization of the ovaries.

A great deal of published work has appeared on the factors which promote fruit swelling after fertilization, but the question why fruit-forming tissues cease to swell at anthesis unless pollinated appears to have been almost entirely neglected. In many plants growth before anthesis of these tissues is largely dependent on cell division, whereas after fertilization cell expansion is the major component of growth, and it is usually postulated that, failing fertilization, cell enlargement ceases because of an inadequate supply of auxin reaching the fruit. If this were so then it would be expected that parthenocarpic fruit could be readily produced by supplying the unpollinated carpel with exogenous auxins, and indeed in a few cases, notably amongst the Solanaceae

and in some Cucurbitaceae, parthenocarpic fruits are easily produced by this means. In the majority of plants, however, it has not been found possible to produce parthenocarpic fruit in this way.

Amongst the plants which are not stimulated to produce parthenocarpic fruits by application of auxin is the strawberry. This is particularly interesting, as work by Nitsch (1950), confirmed by Tukey (1952), showed that after pollination the development of this fruit is closely correlated with auxin produced by the fertilized carpels. They found, for example, that receptacle development was directly proportional to the number of pollinated achenes present. Nitsch (1950) also showed that, if carpels were removed after fertilization, growth of receptacle tissue beneath them ceased; but if a solution of 100 p.p.m. of β-naphthoxyacetic acid in lanolin was supplied to the position previously occupied by the carpel, growth was resumed and fully developed fruits could be produced. He also found (Nitsch, 1955) that after pollination, whereas no free auxin was detectable in the receptacle, the level in the carpel rose rapidly until the 12th day following pollination and then fell. These observations provide strong evidence for his interpretation that the fertilized achenes act as the source of a growth-promoting substance with properties similar to β-naphthoxyacetic acid, which stimulates the development of the receptacle. It has also been shown by Swarbrick (1944) that applications of β-naphthoxyacetic acid to fruits in which pollination was incomplete resulted in larger and more evenly shaped berries.

That the continued development of the strawberry fruit after maturation of the ovaries is not simply dependent upon a supply of auxin was shown by Tukey (1952). In a very extensive series of experiments he treated 1,700 emasculated and unpollinated blooms of more than 60 varieties of strawberry with β-naphthoxyacetic acid, γ-indolylbutyric acid or 3-indolylacetic acid and failed to produce a single parthenocarpic fruit. He found that at anthesis, and for a period subsequently, receptacle tissue which is potentially capable of growth did not respond to auxin applications. This is all the more surprising since, although some cell division took place in the receptacle after pollination, practically all the swelling was due to cell expansion, which is the type of growth usually considered most susceptible to stimulation by applied auxins.

It is the intention in this paper to present observations which it is believed provide evidence that growth of the receptacle ceases at anthesis because of an inhibitory action of the maturing ovaries, and that this inhibition is only relieved by a stimulus resulting from pollination or successful fertilization of the ovaries.

Although the strawberry is a 'false fruit', it may be inferred that the factors governing the swelling of the receptacle are similar to those regulating the swelling of the fleshy parts of true fruits. The accessibility of the carpels, and the large number present, make this a very suitable subject for the study of the influence of the carpels on fruit development, as the effects of their removal or non-pollination can readily be studied both qualitatively and quantitatively.

EXPERIMENTAL RESULTS

Results of imperfect natural pollination. If a single carpel is fertilized (Fig. 1), swelling is restricted to a small part of the receptacle tissue centring on the point of insertion of the achene and hardly extending into the receptacle tissue beneath neighbouring unfertilized carpels. Even where a majority of carpels is fertilized the stimulus does not extend into tissue beneath unfertilized ones, and deep clefts may be produced in the surface of the fruit as a result of the failure to swell of receptacle tissue in these places.

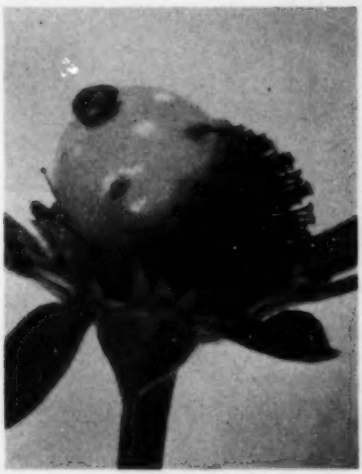

FIG. 1

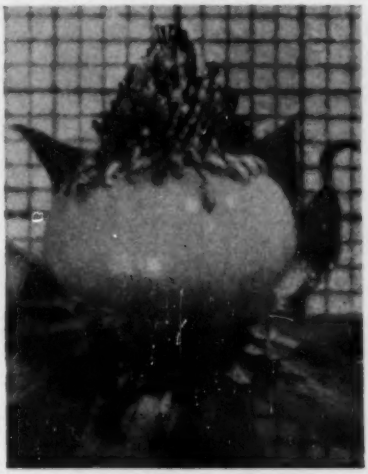

FIG. 2

Fig. 1. Localization of stimulus resulting from the pollination of a single carpel.
Fig. 2. Response of unpollinated fruit of 'Redgauntlet' to applications of gibberellic acid. No swelling occurred beneath the unfertilized carpels.

This raises the question whether the localization of swelling can be explained entirely in terms of a falling concentration gradient of some stimulus radiating from the point of insertion of the fertilized carpel, or whether the surrounding unfertilized achenes actively limit the spread of the stimulus.

Treatment with gibberellic acid. Although gibberellic acid has been reported as causing an increase in size of some fruits, its application has not increased fruit size or set when applied to strawberries growing in the field (Guttridge, 1959, unpublished). Under some conditions it has caused modifications to the normal pattern of fruit development, and Fig. 2 shows a fruit which developed on a plant of 'Redgauntlet' treated at weekly intervals with 50 μg. of gibberellic acid applied to a single mature leaf of the plant in a 0·1 ml. drop of water. Swelling is entirely restricted to the area below the carpels, none of which was pollinated, and stops abruptly at the region of carpel insertion. This

strongly suggests that receptacle tissue immediately beneath the unfertilized carpels was unable to respond to gibberellic acid.

In the two examples described, a growth stimulus to a part of the receptacle has been abruptly limited on reaching tissue beneath unfertilized carpels, and it is suggested that this limitation may be the result of a growth inhibition derived from such carpels. To test this proposal it is necessary to produce a

FIG. 3. Parthenocarpic 'fruit' of 'Cambridge Favourite' developing after the formation of bracts in positions normally occupied by carpels.

receptacle bare of carpels which should then be able to swell either as a result of a stimulus from endogenous auxins, or after the application of growth-promoters such as gibberellic acid or indolylacetic acid. Attempts to remove the carpels a few days before the opening of a flower failed, possibly because of the severe damage caused to the surface of the receptacle. However, it was found possible to produce flowers without functional female parts in two separate ways, and in both cases, when carpels were entirely absent, the receptacle swelled and developed as a 'fruit'.

Production of parthenocarpic fruits. Plants of some strawberry varieties ('Redgauntlet' and 'Cambridge Favourite'), if grown through the winter in a warm glasshouse without supplementary lighting, occasionally produce flowers in which normal carpels are replaced by leafy bracts. Fig. 3 shows a 'fruit' which developed from such a flower on a plant of 'Cambridge Favourite'.

Receptacle tissue beneath the bracts swelled and formed a large fruit-like body. In the few places where normal carpels had been formed swelling was restricted to areas bearing fertilized carpels. It is quite clear that the swelling of tissue beneath the bracts was not dependent upon pollination or fertilization of the ovaries, which were entirely absent, nor were special growth

FIG. 4

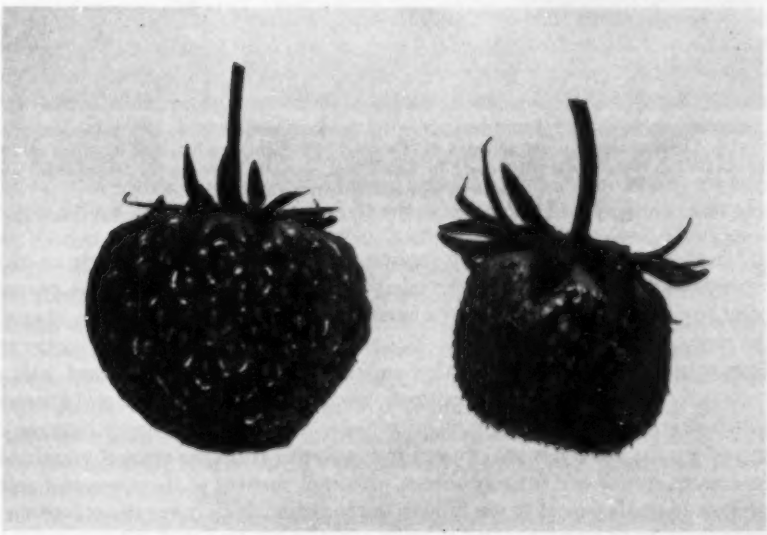

FIG. 5

Fig. 4. Comparison of a normal flower of 'Climax' (on left) with a female sterile flower produced after treatment with maleic hydrazide.

Fig. 5. Comparison of a normal fruit of 'Climax' (on left) with a parthenocarpic fruit developing from a female sterile flower similar to the one shown in Fig. 4.

substances specific to the fertilized carpels necessary. It might be argued that such substances may have been derived from the fertilized achenes at the top of the fruit; but, if so, it would follow that the failure of tissue beneath unpollinated carpels to swell must have been due to an inhibitory effect of these carpels.

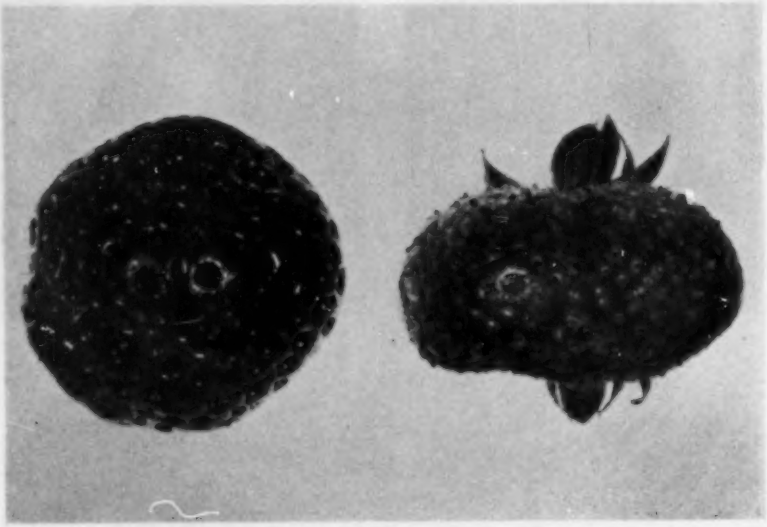

FIG. 6. Comparison of a normal fruit of 'Talisman' (on right), with a fruit in which carpel formation has been locally suppressed by maleic hydrazide. Parthenocarpic development has occurred in areas free of carpels.

An alternative method of preventing the development of carpels on the receptacle is by treatment with maleic hydrazide. Treatment of the parent plant from the time when flower trusses are about three plastochrons old until the time of their emergence from the bud led to the formation of a number of flowers in which carpels were either entirely absent or greatly reduced. Fig. 4 shows a comparison between a healthy flower of the variety 'Climax' and a flower which developed on a plant sprayed at 3-day intervals with maleic hydrazide at 125 p.p.m. No styles are present in the flower from the treated plant and functional carpels are entirely absent, although vestiges of them are probably present as small sacs of tissue hidden in the hairs which cover the receptacle, since these were found in similar fruits which were dissected The size of the receptacle is nearly normal. Some of these female-sterile flowers produced ripe 'fruit' and in Fig. 5 one of these parthenocarpic fruits is compared to a normal fruit of the same variety. As may be seen, it is slightly smaller and rather hairy with a characteristic flattened or rounded end. No achenes of any sort

are visible. Nevertheless, the size was well within the normal range of strawberry fruits and, although rather denser than normal fruits, flavour and colour were very similar.

Thus on the two occasions when carpel formation was prevented in two different ways, the receptacle swelled and parthenocarpic fruit was produced. The absence of carpels was an essential prerequisite for the development of these parthenocarpic fruits, and Fig. 6 shows a comparison between a normal fruit of the variety 'Talisman' and one in which carpel development had been totally suppressed on only part of the receptacle by treatment with maleic hydrazide. Carpels were entirely absent in a narrow band extending from one side of the receptacle to the other, and receptacle tissue along this line swelled; on either side vestigial carpels were present and swelling was restricted, giving a fruit flattened at the sides as illustrated. In several other cases it was found that parthenocarpic development only occurred in areas of the receptacle where carpels were absent or very degenerate.

DISCUSSION

The examples of experimentally induced parthenocarpic fruit in the strawberry described above demonstrate that, when carpels are prevented from forming on the surface of the receptacle, the underlying tissue is able to swell and may develop into a ripe fruit. This must mean that the tissues forming the succulent part of the fruit in the strawberry are not entirely dependent upon fertilized achenes for a supply of growth-promoting hormone. In the case of the parthenocarpic fruit, in which bracts were induced in the place of carpels, the stimulus may have been derived from these bracts; but as no such source of stimulus was available for the parthenocarpic fruits resulting from treatment with maleic hydrazide, they must have obtained any necessary growth-promoting factors from the parent plant—that is, from sources also available to normal fruits. The presence of unfertilized carpels prevented swelling of the receptacle tissue beneath them, and this would suggest that pollination or fertilization results in the removal or suppression of a growth inhibition produced by the carpels as they mature at anthesis. The receptacle tissue is then made susceptible to stimulation by growth promoters produced either in the fertilized carpels or in the parent plant. The fact that parthenocarpic fruits were smaller and denser than normal ones indicated that their auxin supplies may have been inadequate, and this may have been because they lacked that portion normally contributed by the fertilized carpels, as suggested by Nitsch (1950) and Tukey (1952).

The results of experiments performed at the present time do not warrant any discussion of the nature of the postulated inhibitor; whether it is peculiar to the false fruits of the strawberry or widely distributed amongst succulent fruits: or from what part of the ovary it is derived. It is believed that further discussion of its influence and significance in regulating fruit development would be premature until more information is available.

ACKNOWLEDGEMENTS

The author wishes to thank Mr. J. Sunderland for his help in providing the photographs reproduced in this paper.

LITERATURE CITED

NITSCH, J. P. (1950). Growth and morphogenesis of the strawberry as related to auxin. *Amer. J. Bot.* **37**, 211.

—— (1955). Free auxins and free tryptophane in strawberry achenes. *Plant Physiol.* **30**, 33.

SWARBRICK, T. (1944). Progress report on the use of naphthoxyacetic acid to increase the fruit set of the strawberry variety 'Tardive de Leopold'. *Rep. Agric. Hort. Res. Stn. Bristol*, 1943, p. 31.

TUKEY, R. B. (1952). *Some physiological factors influencing the growth and development of strawberry fruits.* Doctoral Thesis, Cornell University.

A Relationship between Gibberellic Acid and Light in the Control of Internode Extension of Dwarf Peas *(Pisum sativum)*[1]

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WITH ONE FIGURE IN THE TEXT

Received 1 November 1960

SUMMARY

Experimental evidence indicates that gibberellic acid (GA) has an insignificant effect on the internode growth-rate in dwarf peas germinated and grown in darkness. When exposed to either red or white light and treatment with GA, dwarf pea seedlings show marked internode extension. Various combinations of light and dark together with GA indicate the possibility of the formation of a light-promoted growth inhibitor which can be reversed in its action by GA. It is suggested that the site for interaction of the postulated inhibitor and exogenous GA is immediately adjacent to the shoot meristem.

INTRODUCTION

GIBBERELLIC acid (GA) brings about marked internode extension in many dwarf species of plants (Brian and Hemming, 1955; Kato, 1955; Bukovac and Wittwer, 1956; Phinney, 1956). The characteristic morphological effects of gibberellin treatment often produce symptoms of etiolation in these species, even when grown under normal light conditions. Lockhart (1956) demonstrated that internode growth of both tall and dwarf varieties of peas is depressed on exposure to red light and that exogenous GA can reverse this effect. From further studies of the effects of red and far-red light on internode extension of dwarf peas (Lockhart, 1958, 1959; Lockhart and Gottschall, 1958) he reached the conclusion that visible radiation probably inhibits stem growth in this dwarf species through an effect on the endogenous level of gibberellin.

The experiments reported in this publication provide further evidence that a relationship exists between the actions of GA and light in the control of internode growth in dwarf peas.

MATERIALS AND METHODS

Dwarf peas (var. 'Meteor') were germinated in moist sand in trays and grown under glasshouse conditions for 6 days, at which time the experimental treatments commenced. After washing the roots free of sand, seedlings were

[1] This work forms part of a thesis approved for the degree of Ph.D. in the University of London.

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transferred to the perforated polythene covers of light-proofed glass jars, so that the roots dipped into 350 ml. of Long Ashton nutrient solution prepared after the manner of Hewitt (1952). Four seedlings were placed in each jar; each treatment comprised three such replications. Growth-regulator treatments were made by dissolving the compounds in the standard nutrient solution to give in each case a concentration of 5 μg./ml.

The various treatments and their controls were then placed on the floor of a plant growth room (described by Selman and Foster, 1957) in a randomized block pattern at an average distance of 7 feet from the light source of twenty 'New Warm White' 80 W. fluorescent tubes. Temperature was maintained at 65° F. Humidity was uncontrolled. Light was kept continuous in the growth room, and dark treatments were made by covering the particular groups of jars with ventilated light-proof cardboard boxes.

In the analysis of weights of material, over-drying was for 12 hr. at 110° C. Ashing was carried out for 3 hr. in a muffle furnace held at 600° C. For the fresh-weight determination of roots the material was first dried by gently pressing between filter-paper.

For the purposes of statistical analysis the main experiment was designed as a factorial experiment with two factors, each containing different levels of treatment.

A. *Growth regulators*

1. Gibberellic acid (5 μg./ml.) in nutrient solution.
2. Indene-1-carboxylic acid (InCA) (5 μg./ml.) in nutrient solution.
3. Control—nutrient solution.

B. *Light–dark treatments*

1. Darkness for 6 days (DD).
2. Darkness for 3 days followed by light for 3 days (DL).
3. Light for 6 days (LL).
4. Light for 3 days followed by darkness for 3 days (LD).

At the end of the 6-day experiments the following measurements were made on the seedlings from each of the twelve combinations of the above treatments:

1. Total height of epicotyl and lengths of each of the first four internodes.
2. Fresh weight, oven-dry weight, and ashed weight, separately, of the roots and shoots.

Each of the variables was analysed using the analysis of variance to determine the effectiveness of the main treatments of growth regulators and light–dark combinations, and to show any interactions that might have occurred between them. Only the analyses of variance which demonstrate interactions are presented in the experimental results: least significant differences ($P = 0{\cdot}05$, $P = 0{\cdot}01$) between treatment means are used to summarize the effectiveness of other treatments.

EXPERIMENTAL RESULTS

A preliminary experiment established that when GA is sprayed on to dwarf peas germinated and grown in complete darkness, there is no significant internode extension comparable to that induced by GA in seedlings of the same age grown under the same conditions but exposed to weak red light.

Dwarf peas were germinated in moist sand in bins which held approximately 200 seeds. Each bin was covered with a glass plate to prevent loss of moisture and either exposed to weak red light from a red-coated tungsten bulb or covered with three layers of black, light-proof cloth. All treatments were held in a constant-temperature room maintained at 25° C. After 130 hrs. from planting the seeds, one-half of the plants in each bin were sprayed with

TABLE I

The growth response of dwarf peas, 48 hrs. after treatment with gibberellic acid, under conditions of darkness and red light

		Internode length (cm.)				
		One	Two	Three	1+2+3	Epicotyl (cm.)
Dark	Control	9·56	6·62	0·52	16·70	16·19
	GA	8·90	7·37	0·81	17·80	17·25
Red light	Control	2·67	2·46	0·96	6·09	7·42
	GA	3·09	4·68	1·73	9·50	11·00
$P = 0·05$		0·73	0·77	0·32	—	1·03
0·01		0·96	1·02	0·42	—	1·36

20 ml. of a water solution containing 20 μg./ml. of GA. The control half of each bin was protected from spray by a screen, and with some practice it was found possible to spray plants uniformly in complete darkness without lateral contamination to control plants.

Seedlings grown in complete absence of light were already about 10 cm. tall at 130 hrs. as against 2 cm. for those grown in red light. Following treatment with GA, plants were maintained under the same conditions for a further 48 hrs., at which time mean values of total height and internode length were measured on groups of 25 plants selected at random. The results are shown in Table I, from which it is readily apparent that GA has a negligible effect on internode extension in the seedlings grown in complete absence of light. The total height of the control plants grown under darkness is apparently shorter than the summation of the first three internodes owing to bending of the epicotyl hook.

Control seedlings grown in darkness showed remarkable elongation of the first and second internodes which together make up most of the structure of the epicotyl. By contrast, those seedlings exposed to light showed the dwarf habit of growth, with much shortened first and second internodes and a very noticeable expansion of leaves. Leaf expansion was uninfluenced by GA in the light-grown seedlings, but GA significantly offset the stunting effect of

red light on the internodes. This is particularly noticeable in the second and third internodes.

The effect of white light is also to inhibit elongation of internodes in dwarf peas (Simpson, 1959) and GA can bring about reversal of this inhibition. The results of a more complex factorial experiment using white light in place of red, and various light–dark combinations, show that GA is dependent on white light for maximum growth activity in internode extension (Tables II–V).

TABLE II

The effects of growth regulators on the growth of dwarf peas treated for six days under different light–dark conditions

(Lengths in cm.; weights in g.)

	Control	GA	InCA	$P = 0.05$	$P = 0.01$
Total height, shoot	11·40	28·80	10·15	0·72	0·98
Internode lengths:					
One	1·26	1·25	1·28	—	—
Two	1·18	1·75	1·17	0·18	0·24
Three	2·35	5·80	2·01	0·38	0·51
Four	2·35	8·35	1·98	0·27	0·36
Fresh weight:					
Shoots	2·800	4·389	2·327	0·247	0·335
Roots	1·667	1·510	1·827	0·166	0·226
Total	4·467	5·899	4·154	—	—
Oven-dry weight:					
Shoots	0·271	0·348	0·229	0·021	0·029
Roots	0·130	0·116	0·144	0·016	0·022
Total	0·401	0·464	0·373	—	—
Ash weight:					
Shoots	0·025	0·029	0·018	0·002	0·003
Roots	0·053	0·047	0·046	—	—
Total	0·078	0·076	0·064	—	—

In this factorial experiment InCA was included as well as GA for two reasons: (*a*) its similarity in structure to the central part of the GA molecule made it possible that it would show gibberellin-like activity, thus enabling a pin-pointing of the active part of the GA molecule, and (*b*) it was desirable to have more than one growth regulator in the factorial type of experiment to facilitate a more rigorous statistical analysis of results.

Table II summarizes the average effects of GA and InCA on the various dimensions and weights of the dwarf peas. Growth-regulator treatment means are obtained as an average from the four different light régimes, and thus any possible interactions between light–dark treatments and growth regulators are concealed.

GA treatment brings about significant height increases in all but the first of the internodes. Total height of the GA-treated plants is nearly treble that of the controls, and the same compound increases both the fresh and oven-dry weights of the entire plant when compared with controls. At the same time

GA causes a change in the root–shoot distribution, favouring translocation to the shoot; this is indicated by higher fresh and oven-dry weights in aerial parts of the plants. InCA gave an opposite effect to GA in the above respects. Ash values remain relatively constant except in the case of InCA treated plants, which have lower values than the others for the shoots.

Table III shows the effect of the light–dark treatments, which are averages from plants treated with GA and InCA and control plants. Any possible inter-

TABLE III

The effects of periods of light and dark on the growth of dwarf peas treated for six days with growth regulators

(Lengths in cm.; weights in g.)

	D. 6 days	L. 6 days	D. 3 days / L. 3 days	L. 3 days / D. 3 days	$P = 0.05$	$P = 0.01$
Total height, shoot	20·34	16·87	14·59	15·35	0·83	1·13
Internode lengths:						
One	1·24	1·41	1·21	1·19	—	—
Two	1·49	1·30	1·40	1·28	—	—
Three	4·67	2·66	3·42	2·79	0·43	0·58
Four	6·89	2·87	2·93	4·20	0·31	0·42
Fresh weight:						
Shoots	3·508	3·544	2·917	2·718	0·278	0·378
Roots	1·593	1·915	1·584	1·580	0·191	0·259
Total	5·101	5·459	4·501	4·298	—	—
Oven-dry weight:						
Shoots	0·255	0·352	0·277	0·248	0·025	0·034
Roots	0·119	0·145	0·133	0·121	0·018	—
Total	0·374	0·497	0·410	0·369	—	—
Ash weight:						
Shoots	0·022	0·034	0·021	0·020	0·002	0·003
Roots	0·044	0·051	0·055	0·047	—	—
Total	0·066	0·085	0·076	0·067	—	—

actions between growth regulators and light–dark treatments are again concealed within the means.

DD for 6 days obviously favours maximum elongation of internodes, and thus total height of the epicotyl, when compared with all other treatments. LL for 6 days, however, favours an increase in the whole plant of fresh, oven-dry, and ashed-weight values as compared with DD grown plants. When darkness and light alternate over 3-day periods the pattern of fresh, oven-dry, and ashed-weight values finally attained is essentially similar, but there is a marked increase in the extension of the fourth internode in those plants which initially have a 3-day period of light, as compared with those that have darkness first.

Although the analyses of variance show that either growth regulators alone or light–dark treatments alone have very significant effects on the variables

that were measured, only 2 of the 11 variables show significant interactions between growth regulators and light–dark treatments. The two interaction effects noted are for the total height of plants and the length of the fourth internode, and the extent of these interactions can be respectively seen in Tables IV and V, together with the associated analyses of variance.

TABLE IV

Total height (cm.) of Meteor pea seedlings after treatment with gibberellic acid (5 μg./ml.) or indene-1-carboxylic acid (5 μg./ml.), and exposure to various combinations of light and dark

Table of means

	Light–dark treatments				Means (solutions)
	D. 6 days	L. 6 days	D. 3 days / L. 3 days	L. 3 days / D. 3 days	
Gibberellic acid	31·85	31·49	24·14	27·73	28·80
Indene-1-carboxylic acid	13·49	9·47	9·21	8·42	10·15
Control—nutrient	15·68	9·66	10·41	9·85	11·40
Means (light–dark)	20·34	16·87	14·59	15·33	

Significant differences	$P = 0.05$	$P = 0.01$
(*a*) *between margin means*		
Solution treatments	0·72	0·98
Light–dark treatments	0·83	1·13
(*b*) *within table means*	1·44	1·97

Analysis of variance

Effects	Degrees of freedom	Variance ratio	'F' value 5%	'F' value 1%	Significance
Light–dark	3	80·74	3·05	4·81	**
Solutions	2	1792·23	3·43	5·61	**
Interaction	6	14·34	2·55	3·70	**

(Variance ratio significant at 5%*, 1%**)

Table IV shows that a highly significant interaction occurs between growth-regulator treatments and light. From inspection of the means within the table it is apparent that, within the gibberellin treatments, the two means which show the effect of 3-day light treatments given in the first half of the experiment (LL, 31·49; LD, 27·73), are proportionately much higher than those in control and InCA treated plants. Table V shows that part of the interaction effect between GA and the initial 3-day light period on the total height of the plant can be accounted for in the extra growth of the fourth internode.

In many of the plants internodes above the fourth node were very condensed because of proximity to the meristematic region, and individual measurements were omitted. An estimate of these internode lengths was obtained as an average figure by subtracting the summation of the first four internodes from the figure for the total height of a plant (top shaded area of

TABLE V

Mean extension (cm.) of the fourth internode of Meteor peas after treatment with gibberellic acid (5 μg./ml.) or indene-1-carboxylic acid (5 μg./ml.), and exposure to various combinations of light and dark

Table of means

Solution treatments	Light–dark treatments: D. 6 days	L. 6 days	D. 3 days L. 3 days	L. 3 days D. 3 days	Means (solutions)
Gibberellic acid	11·44	6·20	6·39	9·38	8·35
Indene-1-carboxylic acid	4·16	1·18	1·09	1·48	1·98
Control—nutrient	5·08	1·23	1·33	1·74	2·35
Means (light–dark)	6·89	2·87	2·93	4·20	

Significant differences	$P = 0.05$	$P = 0.01$
(*a*) *between margin means*		
Solution treatments	0·27	0·36
Light–dark treatments	0·31	0·42
(*b*) *within table means*	0·53	0·72

Analysis of variance

Effects	Degrees of freedom	Variance ratio	'F' value 5%	'F' value 1%	Significance
Light–dark	3	323·06	3·05	4·81	••
Solutions	2	1559·10	3·43	5·61	••
Interaction	6	19·11	2·55	3·70	••

(Variance ratio significant at 5%•
1%••)

each histogram, Fig. 1). Fig. 1 clearly shows the remarkable growth response of internodes above the fourth node following GA treatment together with a 3-day period of light in the first half of the experimental period.

DISCUSSION

The experiments using GA and light–dark régimes clearly indicate that the gibberellin-stimulated growth response is dependent on light and also that the site of the synergism is close to the shoot tip. It would seem likely that the presence of the shoot tip is essential to the gibberellin effect on growth.

The dependence of the GA growth reaction on light was further demonstrated when dwarf peas were germinated and grown in complete darkness. These dwarf peas showed a remarkable degree of internode elongation quite independent of, and not increased by, GA. In order to interpret this in terms of the widely held theory of auxin destruction in light, it might be supposed that, in darkness, adequate supplies of such a growth regulator as IAA are present; but that in plants exposed to light photo-destruction of IAA occurred, which limited the possible growth. On this basis, the growth response from GA applied in light could then be considered as 'protective', preventing photo-destruction of IAA. Such a theory could perhaps explain the poor

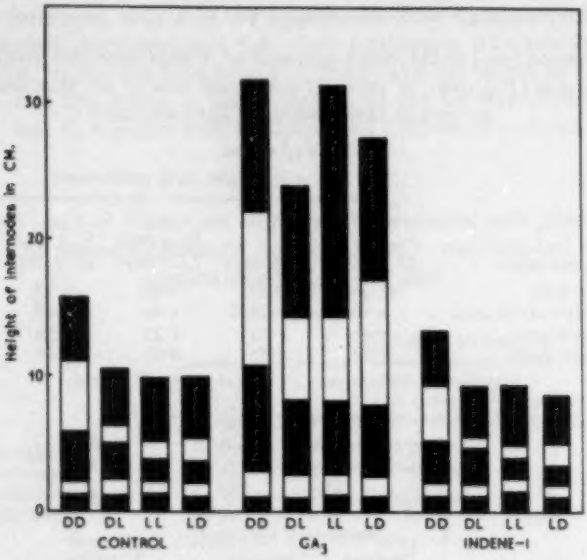

FIG. 1. The response of individual internodes of 'Meteor' peas to gibberellic and indene-1-carboxylic acids when exposed to different periods of light and dark for 6 days.

DD = darkness for 6 days.
DL = dark for 3 days, followed by light for 3 days.
LL = light for 6 days.
LD = light for 3 days, followed by dark for 3 days.

Internodes are represented by alternate black and white bars. The fifth internode, the shaded area at the top of each column, represents the difference between the total height of the plant and the sum of the first four internodes.

activity shown by GA alone in standard straight-growth tests for auxins, where endogenous growth promoters are usually in low supply or absent, a fact which at least indicates that GA may rely for its activity on the presence of some other factor.

On the other hand it could equally well be postulated that an actively growing pea seedling (tall or dwarf) is adequately supplied with growth stimulators in darkness or light, and that a growth inhibitor is produced in the light (large amounts in the dwarf, smaller in tall peas) which can be blocked at its site of action by an exogenous supply of GA. Thus GA would not need to have growth-stimulating activity *per se*, provided it had a molecular structure which could 'fit' more easily at the site of action of the inhibitor than could the naturally occurring inhibitor itself. For such competitive antagonism to operate, however, the two molecules would have to be closely similar, as in the case for example when α-(4-chlorophenoxy)-*iso*butyric acid lowers the activity of 4-chlorophenoxyacetic acid (Wain and Wightman, 1957).

Brian (1958, 1959) has put forward a comprehensive theory in which

gibberellin action is attributed to the neutralization of a growth-inhibitory system which can limit growth even though auxin is present in non-limiting amounts. It was suggested by him that in response to light, gibberellin-like hormones are formed in leaves, a physiologically inactive precursor being an intermediary. In darkness the active hormone is converted slowly back to the precursor, this change occurring more rapidly if the leaf is exposed to far-red light. The theory implies the continual presence of an inhibitor, growth being controlled by fluctuations in the gibberellin supply.

However, from the evidence presented in this paper it is clear that, at least in dwarf peas, maximum natural growth stimulation takes place in darkness, at a time when, according to the theory of Brian, any natural gibberellin in the plant is either in the inactive form or altogether absent. The blocking of the postulated inhibitor would therefore seem to be impossible at this stage. We must therefore assume that either there is no natural growth inhibitor present in darkness, or that naturally occurring GA is active both in darkness and the light. The second would confound the theory of Brian; and if we accept the first assumption we must conclude that an inhibitor is produced in light since, in the Brian theory, the action of the naturally occurring gibberellin is to neutralize an inhibitor.

It has also been demonstrated in the present paper that an exogenous supply of GA to intact dwarf peas is extremely effective in promoting growth under white light. This fact implies either a limited supply of endogenous gibberellin, complete absence of endogenous gibberellin, or the presence of an inhibitor in quantities greater than the naturally occurring gibberellin.

In the opinion of the writers, it is far easier to explain the dependence of exogenous GA on light, and the remarkable growth of dwarf peas in darkness, by postulating that the plant produces an inhibitor in light which can be blocked in its action by an exogenous supply of GA. Such a simple theory would take into account the likelihood that IAA is a major natural growth promoter in plants and that it is present in optimum quantities at all times. However, until further chemical evidence for a light-promoted growth inhibitor is forthcoming, the above theory cannot be substantiated.

LITERATURE CITED

BRIAN, P. W. (1958). Role of gibberellin-like hormones in regulation of plant growth and flowering. *Nature* (London), **181**, 1122–3.

—— (1959). Effects of gibberellins on plant growth and development. *Biol. Revs.*, **34**, 37–84.

—— and HEMMING, H. G. (1955). The effect of gibberellic acid on shoot-growth of pea seedlings. *Physiol. Planta.* **8**, 669–81.

BUKOVAC, M. J., and WITTWER, S. H. (1956). Gibberellic acid and higher plants. *Quart. Bull. Mich. agric. Exp. Sta.* **39**, 307–20.

HEWITT, E. J. (1952). *Sand and water culture methods.* Tech. Commun. 22. Commonwealth Bureau of Horticultural and Plantation Crops. C.A.B., 187–190.

KATO, J. (1955). Responses of plant cells to gibberellin. *Bot. Gaz.* **117**, 16–24.

LOCKHART, J. A. (1956). Reversal of the light inhibition of pea stem growth by the gibberellins. *Proc. nat. Acad. Sci., Wash.*, **42**, 841–8.

—— (1958). The influence of red and far-red radiation on the response of *Phaseolus vulgaris* to gibberellic acid. *Physiol. Planta.* **11**, 487–92.

LOCKHART, J. A. (1959). Studies on the mechanism of stem growth inhibition by visible radiation. *Pl. Physiol.* **34**, 457–60.

—— and GOTTSCHALL, V. (1958). Growth response of *Pisum* in relation to light and gibberellic acid. *Pl. Physiol. Supp.* **33**, xl.

PHINNEY, B. O. (1956). Growth response of single gene dwarf mutants in maize to gibberellic acid. *Proc. nat. Acad. Sci., Wash.*, **42**, 185–9.

SELMAN, I. W., and FOSTER, J. C. (1957). Wye College plant growth rooms. *J. Hort. Sci.* **32**, 49–52.

SIMPSON, G. M. (1959). *Studies on the occurrence and distribution of certain growth hormones in plants.* Ph.D. Thesis, London University.

WAIN, R. L., and WIGHTMAN, F. (1957). Studies on plant growth-regulating substances. XI. Auxin antagonism in relation to a theory on mode of action of aryl- and aryloxyalkanecarboxylic acids. *Ann. appl. Biol.* **45**, 140–57.

Effect of Light Quality on the Growth and Growth-substance Content of Plants

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WITH FOUR FIGURES IN THE TEXT

Received 23 September 1960

SUMMARY

The main effect of incandescent light on most of several species and varieties of plants grown in cabinets illuminated by fluorescent light was to increase the length of internodes. Although leaf area was little affected, incandescent light increased the dry weight of most plants.

In dwarf French bean plants, the longer internodes of plants grown with additional incandescent light was associated with, and perhaps a result of, the higher content of gibberellic acid-like and indolylacetic acid-like growth substances.

INTRODUCTION

A LIGHT intensity and spectral energy distribution similar to sunlight are difficult to obtain in a controlled environment by artificial illumination. The excessive heat produced by tungsten filament lamps can be largely avoided by using fluorescent tubes, but the original 5 ft. 80 W. tubes did not give an adequate intensity. Intensity has since been increased by internal reflector tubes, greater efficiency, and increased watts per foot, but the spectral energy distribution curves for 'White 3,500° K' fluorescent tubes (Atlas, 1957) shows very little radiation with wavelength greater than 720 mμ, which the Dutch Committee on Plant Irradiation (1953) report to have a specific effect in promoting elongation of stems. This deficiency in the red and infra-red part of the spectrum can be made up by incandescent lamps emitting a high proportion of their energy in the region 720–1,000 mμ.

The experiments here described were made first to see what effects additional incandescent light has on plants grown under fluorescent light in cabinets. An explanation of the effects on dwarf French bean was then sought by analysis for growth-promoting substances.

METHODS

Thirteen 8 ft. 125 W. internal reflector fluorescent tubes, with space for four 4 ft. 150 W. incandescent striplights between them, were supported on a movable frame in each of two cabinets in a well-ventilated, thermostatically controlled room. The amount of supplementary incandescent light was varied by operating the two striplights, in each half cabinet, in parallel when the watts consumed by the pair of striplights was 300 W. or in series when they fell to 75 W. This incandescent light increased the light intensity, measured

50 cm. below the lights by a selenium-cell foot-candle meter, from 1,400 to 1,420 f.c. but a radiation meter, measuring the total radiation between 400 and 3,000 mμ, showed an increase from 5·5 to 11·6 mW./cm.^{2} with full incandescent light. Light intensity at plant level was varied by vertical movement of the light frame.

The temperature in the room was maintained at 20±0·9° C. as the photoperiods alternated in each cabinet. The temperature in each cabinet, measured with screened resistance thermometers (Long, 1957) at pot level, was 1·5° C. higher with the lights on although air was drawn by a fan first between the plants then between the lights. Humidity was not controlled, but excess water vapour was condensed by refrigeration. Water-supply was controlled by weighing marked pots from each treatment, watering to constant weight, and then watering the replicates similarly. Carbon dioxide was kept at atmospheric concentration by continuous ventilation. Without ventilation the CO_2 concentration, measured by an infra-red gas analyser, increased when the lights were on, presumably because of respiration in the soil, but increased more in the dark.

Plant-growth substances in the tissues were extracted, purified, and separated on paper chromatograms with isopropanol: ammonia solution: water [10:1:1], by previously described methods (Wheeler, 1960). Zones on chromatograms corresponding to indolylacetic acid (IAA) and gibberellic acid (GA) marker spots were assayed respectively by the growth of 10-mm. sections cut from wheat coleoptiles 3 days old and 6-mm. disks cut from the etiolated primary leaves of dwarf French bean plants 6 days old. The sections and disks were measured after incubation for 22 and 24 hours respectively at 25°C. in darkness.

RESULTS

Growth of dwarf and tall varieties of French bean (Phaseolus vulgaris), *broad bean* (Vicia faba) *and garden pea* (Pisum sativum) *and of a runner bean* (Phaseolus multiflorus). The various varieties of bean and pea (abbreviations used are given in parenthesis) were grown for 20 days in polythene pots containing 500 g. of John Innes potting compost: dwarf French bean, var. Sutton's selected Canadian Wonder (DFB); climbing French bean, var. Sutton's Earliest of All (CFB); runner bean, var. Sutton's Scarlet (RB); broad bean, var. Sutton's Dwarf (DBB); broad bean, var. Sutton's Exhibition Longpod (TBB); dwarf garden pea, var. Meteor (DP); and garden pea, var. Sutton's Improved Pilot (TP).

Fresh and dry weights and heights of DFB, CFB, RB, and DP (except RB height) increased with increasing incandescent light (Table I), whereas those of DBB decreased and those of TP or TBB were unaffected. The area of the primary leaves (first pair of opposite leaves above the cotyledons) of the French and runner bean and of the third leaf of pea and broad bean was unaffected by incandescent light but the area of the middle leaflet of the first trifoliate leaf of French and runner bean increased.

TABLE I

Growth of plants in fluorescent light alone and with additional incandescent light for 20 days

	Watts*	DFB	CFB	RB	DBB	TBB	DP	TP
Mean fresh weight (g.)	0	9·6	3·9	12·1	3·9	7·8	1·7	3·7
	75	11·0	4·2	13·4	3·1	8·3	1·7	4·5
	300	12·3	7·0	13·6	2·7	8·8	2·1	3·7
Mean dry weight (g.)	0	0·98	0·36	1·33	0·39	0·67	0·18	0·45
	75	1·08	0·38	1·38	0·31	0·87	0·18	0·56
	300	1·17	0·68	1·50	0·27	0·79	0·21	0·47
Mean height (mm.)	0	117	130	882	71	224	69	417
	75	141	213	741	58	214	75	492
	300	193	408	874	56	222	82	424
Mean leaf area† ($cm.^2$)	0	117	54	179	16	34	7	15
	75	121	53	164	12	33	8	15
	300	119	75	181	12	35	8	13

* Watts of incandescent lighting additional to 813 W. of fluorescent lighting.
† Mean leaf area of one primary leaf of French and runner bean and one leaflet of third leaf of broad bean and pea.
See text for explanation of plant name abbreviations.

The pots were arranged in three Latin squares under three levels of incandescent lighting, viz. 0, 75, and 300 W. The light intensity varied along the length of the fluorescent tube, decreasing towards its ends. To increase differences in intensities, plants at the centre were placed nearer the fluorescent lights than plants at the ends. Plants in different positions in the direction across the tubes did not differ significantly, indicating uniformity of lighting, but those in different positions along the length of the tubes had different fresh and dry weights, decreasing as the light intensity decreased, whereas their heights and leaf areas did not differ significantly.

To test for varietal differences in sensitivity to incandescent light, five varieties of dwarf French bean [Sutton's Selected Canadian Wonder (CW), The Prince (TP), Phenix Claudia (PC), Sutton's Golden Waxpod (GW), and Comtesse de Chambord (CC)] were grown with illumination by fluorescent and incandescent lamps; one batch was illuminated for 16 hours (LD) and the other for 8 hours (SD) per day. Fresh and dry weights and height increased with incandescent light (Table II). After 14 days, plants with SD were taller and after 26 days were shorter than with LD treatment. The interaction of daylength and incandescent light on height was highly significant ($P<0.001$) after 26 days. Short days decreased fresh and dry weights but the interaction of daylength and incandescent light was not significant. Primary leaf area increased slightly with incandescent light after 14 days but not after 26 days. Short days decreased primary leaf area.

TABLE II

Growth of dwarf French bean varieties in fluorescent light alone (F) *and with additional incandescent light* (F+I) *for 26 days*

	Lighting	Daylength*	CW	TP	PC	GW	CC	S.E.
Mean fresh weight (g.)	F	LD	17·6	14·5	12·8	12·1	12·0	
	F+I	LD	17·0	16·0	14·8	13·1	13·1	
	F	SD	10·7	11·1	8·6	7·9	5·8	
	F+I	SD	10·3	11·6	8·8	9·3	7·1	0·62
Mean dry weight (g.)	F	LD	1·92	1·70	1·55	1·41	1·25	
	F+I	LD	1·93	1·94	1·81	1·58	1·43	
	F	SD	1·11	1·10	0·88	0·78	0·51	
	F+I	SD	1·15	1·21	1·00	0·99	0·65	0·070
Mean height (cm.)	F	LD	143	135	140	104	126	
	F+I	LD	170	193	170	137	162	
	F	SD	145	135	139	104	87	
	F+I	SD	142	150	142	119	111	6·9
Mean primary leaf area ($cm.^2$)	F	LD	120	137	85	85	48	
	F+I	LD	111	136	93	100	48	
	F	SD	93	110	81	76	39	
	F+I	SD	93	127	75	89	45	5·9

* Daylength 16 hours (LD) or 8 hours (SD).
See text for explanation of variety abbreviations.

Experiments were made with barley (var. Proctor), sugar-beet (var. PBI–KNB), kale (var. Thousand-headed), radish (var. Sutton's Red White-tipped), potato (var. King Edward) and tobacco (var. White Burley) but effects of incandescent light on fresh and dry weights and leaf areas were different in different experiments and so are not reported. Possible explanations of this inconsistency are discussed later. The height of kale, potato, and tobacco stem was increased consistently by incandescent light (Fig. 1).

Plant-growth substances in dwarf French bean internode extracts. Internodes immediately above the node bearing the primary leaves of dwarf French bean (var. Sutton's selected Canadian Wonder), grown for 14 days under fluorescent and incandescent light, were extracted with ethyl acetate (Wheeler, 1960). The fraction of the extract containing acidic substances was divided equally between two spots on one chromatography paper. One chromatogram was assayed by wheat coleoptile sections and the other by etiolated dwarf bean leaf disks (Fig. 2). Assay by wheat coleoptile sections, the growth of which is sensitive to auxins, indicated most growth promotion in the region of the chromatogram similar to the IAA marker spot. In the assay by etiolated dwarf bean leaf disks, sensitive to gibberellins, the only significant effect was growth promotion in the region of the chromatogram corresponding to the GA marker spot.

Comparison of growth and growth-substance content of dwarf French bean internodes grown in fluorescent light with or without the addition of incandescent

light. To accentuate the increase in height caused by incandescent light only five fluorescent tubes were used per cabinet instead of thirteen. Thus in each half cabinet the fluorescent tubes consumed 312·5 W. with an additional 300 W. consumed by the incandescent lights. The incandescent light increased the light intensity only from 800 to 870 f.c. at 30 cm. below the tubes.

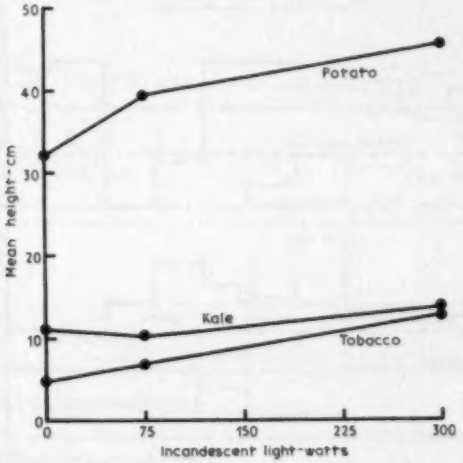

FIG. 1. Effect of incandescent light on stem height of potato, kale, and tobacco.

Incandescent light affected the growth-rate of the hypocotyls and epicotyls only slightly but the growth-rate of the next three internodes increased with additional incandescent light (Fig. 3). Extracts of these three internodes were made and assayed after 14 and 20 days' growth.

As the auxin Rf 0·31–0·48 and the gibberellin Rf 0·50–0·71 were separated on the paper they could be assayed on the same chromatogram by the wheat coleoptile section test and etiolated dwarf bean leaf disk test respectively. The GA-like growth-substance content of the internodes increased with incandescent light but there was no significant change with age (Fig. 4A). The IAA-like growth-substance content of the internodes increased significantly with the addition of incandescent light and even more with increasing age of the plants (Fig. 4B). In neither assay did incandescent light and age interact significantly.

The primary leaves of dwarf French bean plants at 7 days old had similar leaf areas and similar contents of gibberellin whether given incandescent light or not.

DISCUSSION

Increase in stem height by incandescent light was reported by Wassink and Stolwijk (1956) and van der Veen and Meijer (1959) and their work is extended in this communication. Elongation was greatest in dwarf French

bean internodes above the epicotyl. Incandescent light increased air and leaf temperatures, light intensity, and the proportion of red and infra-red radiation. Air temperature in the cabinet was raised only 0·5° C. by the incandescent light but the increase in leaf temperature was probably greater (Ansari and Loomis, 1959). Increased temperature may explain small differences, especially where

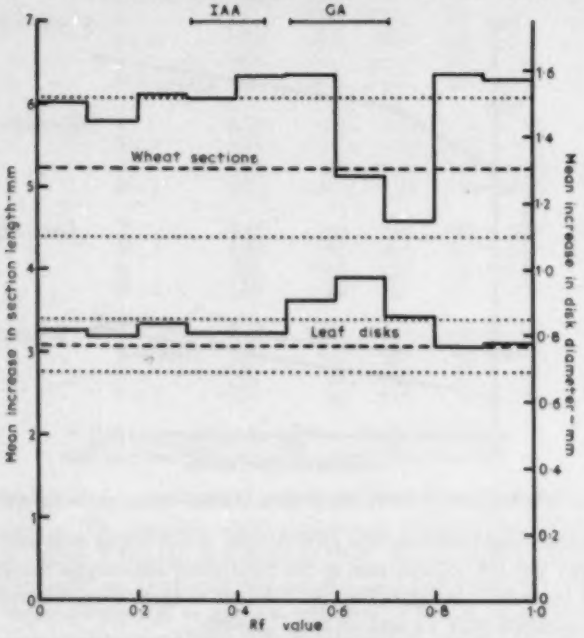

FIG. 2. Assay of chromatograms of the fraction of dwarf French bean internode extracts, containing acidic substances, by 6-mm.-diameter etiolated dwarf French bean leaf disks and 10-mm. wheat coleoptile sections. Growth of controls in each test is indicated by a broken line on either side of which three times the standard error of the means is indicated by dotted lines. Horizontal lines at the top of the figure indicate positions of the marker spots. Each histogram is the mean of two assays.

the initial rate of growth was increased but final size was not affected, e.g. primary leaf area of dwarf French bean. Increased light intensity with additional incandescent light was small, even when the incandescent lamps consumed as many watts as the fluorescent tubes. Growth of dwarf French bean is little influenced by light intensities above 1,000 f.c. (Leiser, Leopold, and Shelley, 1960). The increased amount of red and infra-red radiation, or their increased proportion to white light, probably caused the internodes to elongate.

Increased height of plants in incandescent light brought the stem apex closer to the lights and thus to a higher light intensity and temperature. This may explain why the incandescent light affected the trifoliate leaves of dwarf French bean more than the primary leaves.

Dry-matter production depends on the total radiation received by a plant, thus dry weight decreased in short days. Upper leaves, brought nearer to the lights, received more radiation and Table I shows that increased height was

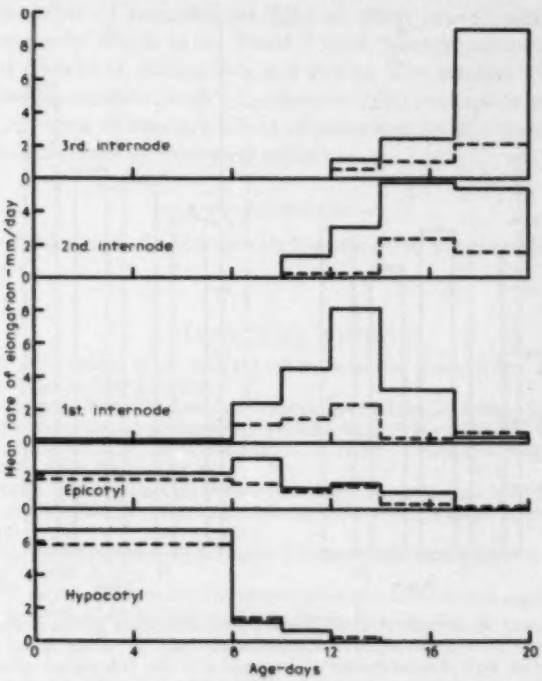

FIG. 3. Effect of fluorescent light alone (- -) and with the addition of incandescent light (——) on the rate of growth of dwarf French bean internodes.

associated with more weight. Plants grown with additional incandescent light required more water, e.g. in one experiment with kale 530 ml. of water was needed to keep pot-weight constant during 21 days' growth under fluorescent light and 580 ml. with incandescent light. Potted plants tend to dry out and wilt somewhat towards the end of some long experiments which may explain lower fresh and dry weights of plants grown with incandescent light, e.g. after 56 days fresh and dry weights of kale and sugar beet were decreased by incandescent light.

The two growth substances behaved on chromatograms and in growth tests like IAA and GA respectively but the evidence was not taken as conclusive identification. In primary leaves of dwarf French bean the maximum content of gibberellin-like leaf-growth substances coincides with the maximum rate of leaf expansion (Wheeler, 1960). The gibberellins in the internodes were possibly similar to those found in the primary leaves, but the two were not

compared directly. In the present experiments the three internodes were sampled together, so any relationship between growth-rate of internodes and their growth-substance content was obscured. The resultant growth-rate of

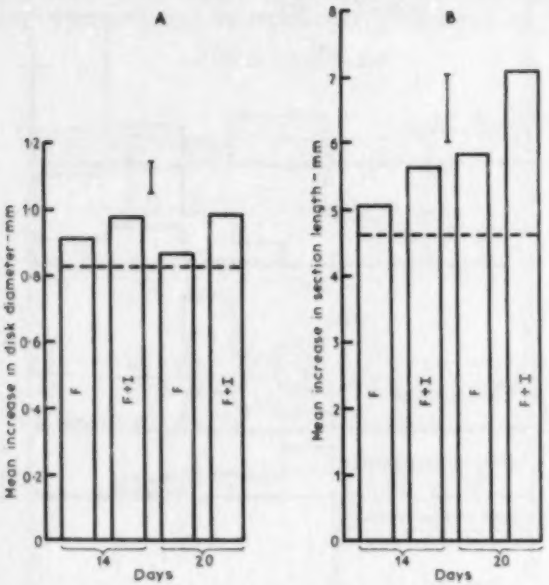

FIG. 4. Assay of extracts of dwarf French bean internodes grown in fluorescent light alone (F) or with the addition of incandescent light (F+I) for 14 and 20 days. The zone on each chromatogram corresponding to the GA marker spot was assayed with 6-mm.-diameter etiolated dwarf French bean leaf disks (A) and the zone corresponding to the IAA marker spot with 10-mm. wheat coleoptile sections (B). Growth of controls in each test is indicated by a broken line. Vertical lines are three times the standard error of the means.

the three internodes was nearly uniform between 12 and 20 days, but was more than three times greater with incandescent light than without, i.e. growth-rate depended on incandescent light and not on age. This implies that the gibberellins were correlated with the rate of extension and not with the total amount of tissue formed. The growth substances may be expressed as a concentration, by means of calibration curves relating growth response to GA or IAA. Between 14 and 20 days under fluorescent light alone or with additional incandescent light, the concentration of gibberellin fell from 34 and 27 to 6 and 7 μg. GA/kg. fresh weight respectively. Thus, concentration fell with age but was not affected by the light régimes. The auxins increased under the incandescent-light treatment and more with age of plant. Expressed as IAA concentration, after 14 and 20 days there was 123 and 81 μg. IAA/kg. fresh weight in fluorescent light alone and 134 and 120 μg./kg. respectively

with additional incandescent light, probably no change in concentration with internode extension. This could be interpreted that auxin content was an effect of growth and not its cause.

The major effect of incandescent light on plant growth was to increase length of internodes which in the dwarf French bean seems to happen from an increased content of gibberellins and auxins. The amount of dry matter produced usually increased with incandescent light, perhaps because of stem elongation; apparent deleterious effects of incandescent light may occur from water stress occasioned by increased radiation.

ACKNOWLEDGEMENT

I wish to thank Dr. E. C. Humphries for advice on the controlled environment facilities.

LITERATURE CITED

Ansari, A. Q., and Loomis, W. E. (1959). Leaf temperatures. *Amer. J. Bot.* **46**, 713.

Atlas. (1957). *Atlas Lighting Catalogue.*

Dutch Committee on Plant Irradiation. (1953). Specification of radiant flux and radiant flux density in irradiation of plants with artificial light. *J. Hort. Sci.* **28**, 177.

Leiser, A. T., Leopold, A. C., and Shelley, A. L. (1960). Evaluation of light sources for plant growth. *Plant Physiol.* **35**, 392.

Long, I. F. (1957). Instruments for micro-meteorology. *Quart. J. Roy. Met. Soc.* **83**, 202.

Veen, R. van der, and Meijer, G. (1959). *Light and plant growth.* Philips' Technical Library, Eindhoven.

Wassink, E. C., and Stolwijk, J. A. J. (1956). Effects of light quality on plant growth. *Ann. Rev. Plant Physiol.* **7**, 373.

Wheeler, A. W. (1960). Changes in a leaf-growth substance in cotyledons and primary leaves during the growth of dwarf bean seedlings. *J. exp. Bot.* **11**, 217.

The Influence of Water Strain on the Minimum Intercellular Space Carbon dioxide Concentration Γ and Stomatal Movement in Wheat Leaves

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Received 7 October 1960

SUMMARY

Eight detached wheat leaves were each subjected to varying degrees of controlled water strain, in a specially constructed leaf chamber (which is described in detail) connected to a closed system incorporating an infra-red gas analyser. The water strain was varied by applying successively to their leaf sheaths two concentrations of mannitol solution (0·2 M. and 0·4 M.) each preceded and followed by water. Conditions were maintained constant for light (900 f.c.), leaf temperature (25° C.) and humidity (50 per cent. RH) of the circulating air as it entered the leaf chamber. Steady-state values (gamma) of the carbon dioxide concentration in the closed system were recorded both for the water and mannitol treatments and the data analysed statistically. The two mannitol treatments were found to increase gamma from 80 p.p.m. with water to 94 and 116 p.p.m. respectively, all differences being highly significant. Concomitant measurements were made of stomatal resistance with a porometer and of transpiration by means of wet and dry thermocouples; the stomata showed marked closure in response to mannitol treatment (complete closure with the higher concentration) and partial reopening with the leaf sheath in water once more, transpiration also falling and rising inversely with water strain.

It is concluded that part of the closing response of wheat stomata to water strain under natural conditions must be operated by increase in the intercellular-space carbon dioxide concentration, but a comparison with previous experimental results suggests that this can only be a small part unless sensitivity to carbon dioxide is greatly increased by water loss.

The *initial* response to mannitol treatment was a preliminary opening of the stomata and a fall of about 10 per cent. in carbon dioxide concentration. These results imply that under natural conditions the preliminary opening of wheat stomata with wilting may be partly passive and partly active with a carbon dioxide-operated mechanism.

A subsidiary experiment with water strain varied by removing and restoring the water supply to the leaf sheath gave increases and decreases in carbon dioxide similar to those in the main experiment; the results of the latter need not, therefore, be attributed to metabolism of the mannitol.

It is suggested that values of gamma give a good measure of the efficiency of the

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mesophyll for net carbon dioxide absorption, with the stomatal factor eliminated, under the given conditions and with carbon dioxide 'limiting' in the sense used by Maskell (1928).

INTRODUCTION

BRIGHTLY illuminated leaves enclosed in a limited volume of air, either with or without carbon dioxide, bring the concentration of that gas to a steady-state value. Work by Miller and Burr (1935) with whole plants and by Gabrielsen (1948) with detached leaves suggested that this concentration was almost constant at about 0·01 per cent. by volume or 100 p.p.m. Heath (1939, 1949, 1951) also found that the concentration of carbon dioxide in air which had been passed through the intercellular space systems of detached illuminated leaves of *Pelargonium zonale* and *Begonia sanguineum* was reduced only to about 0·01 per cent. He interpreted this as the minimum concentration at the surface of the assimilating cells, and put forward as one suggestion 'that the 0·01% CO_2 represents the concentration which must be maintained in the intercellular spaces to give a rate of assimilation high enough to balance the respiration rate; in fact that it is the "compensation point" for CO_2 concentration'. Heath was, however, impressed by the almost constant concentration found for very diverse types of leaf—*P. zonale* has chloroplasts in every epidermal and mesophyll cell while *B. sanguineum* has extensive chlorophyll-free hypodermal layers (Heath, 1941). This seemed difficult to explain on the basis of a simple balance between assimilation and respiration. The name 'carbon dioxide compensation point' has been used also by Egle and Schenk (1953) and Rabinowitch (1951, 1956), for the measured steady-state concentration in a closed system, and *effectively* it is this; but Heath and Orchard (1957) preferred to avoid any implications as to the precise relations of respiration and photosynthesis involved by using the non-committal symbol Γ (gamma) for this concentration. If movement of carbon dioxide through the cuticle of the epidermis is negligible so that effectively all exchange takes place through the stomatal pores, Γ will also be the concentration in the intercellular spaces; hence it will be the minimum possible concentration that can be reached in these spaces (including the substomatal cavities) for a similarly illuminated leaf placed in ordinary air of 0·03 per cent. initial carbon dioxide content. Any factor found to increase Γ in closed system experiments must tend also to increase the substomatal cavity carbon dioxide concentration in the open air, and thus tend to cause stomatal closure (Heath, 1950; Heath and Russell, 1954).

Miller and Burr (l.c.) claimed that Γ was independent of temperature, but the later work of Egle and Schenk (l.c.) and of Heath and Orchard (l.c. and Orchard, 1956) showed that in a wide range of species Γ increased markedly with temperature. In *Coffea arabica* and *Allium cepa*, both species recorded as showing mid-day closure of the stomata, Heath and Orchard (1957) found an especially sharp increase in Γ with temperature above 30° C., the value rising to 240 p.p.m. at 35° C.; in *P. zonale*, for which mid-day closure had not been recorded, Γ increased smoothly with temperature up to 35° C. and there reached

a value of only 120 p.p.m. Later work by Meidner (unpublished) with wheat, another species in which mid-day closure has not been recorded, has indicated very low Γ values with no sudden increase at high temperatures and even at 40° C. a Γ of only 87 p.p.m. (at a light intensity of 600 f.c.). Heath and Orchard (l.c.) suggested that midday closure in onion and coffee was a high-temperature effect acting through the substomatal cavity carbon dioxide concentration. This was supported for onion by Meidner and Heath's (1959) experiments which showed that the closing effect of high temperature was changed to an opening effect if the leaf cavity was continuously swept with CO_2-free air.

Although wheat stomata do not apparently show mid-day closure, and on the above hypothesis might therefore be expected not to close in response to high temperature, they were known to be sensitive to water strain such as could be caused by dry moving air with no restriction of water supply to the roots of potted plants (Heath and Milthorpe, 1950). Using detached wheat leaves, Milthorpe had investigated the effects, upon stomatal movement and transpiration rate, of completely cutting off the water supply and then restoring it (Milthorpe and Spencer, 1957). The present work arose out of preliminary experiments planned to impose much milder and controlled water strain with mannitol solutions. These showed that subjecting wheat leaves to different degrees of water strain by immersing their sheaths in mannitol solutions of different molar concentrations caused marked changes in the Γ values measured under constant light and temperature.

An experiment was therefore designed to investigate the influence of water strain on Γ, with concomitant measurements of stomatal movements and transpiration rates. A preliminary note summarizing the more important results of this experiment has already been published (Heath and Meidner, 1958).

APPARATUS

Except for the leaf chamber assembly and the method of transpiration measurement the apparatus used was essentially that designed and assembled by Orchard (1956), operating on the closed circuit setting and with only detail modification (Meidner, 1958). The whole system was housed in a constant temperature room maintained at 25° C. It is shown very diagrammatically in Fig. 1. The Infra-red Gas Analyser[1] was used at a sensitivity giving full-scale deflection for 0·02 per cent. carbon dioxide; the water-jacketed humidifiers were maintained at a temperature of 12° C., giving a relative humidity of 50 per cent. in air entering the leaf chamber at 23° C. Transpiration was measured with two pairs of wet (*W*) and dry (*D*) thermocouples (cf. Glover, 1941), one pair in the air stream entering the leaf chamber and one pair in the air stream leaving it. Both pairs were in tubes immersed in the water bath at approximately 23° C. (Fig. 1). Each of the four thermojunctions was connected to a single reference junction kept at constant temperature (as was also the thermo-couple inserted in the leaf blade—see below). A multi-pole switch enabled a single ('Scalamp') galvanometer to be used to obtain the absolute values

[1] Grubb-Parsons three-box model.

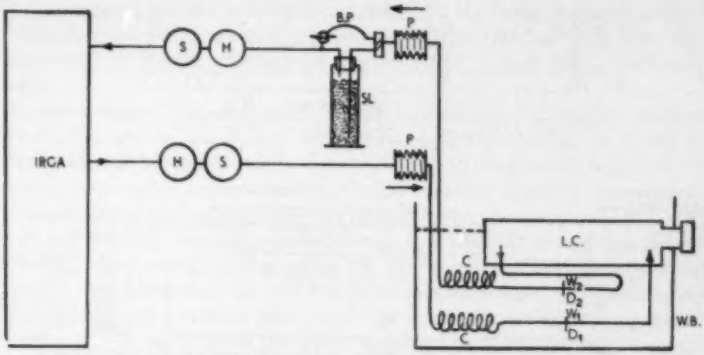

FIG. 1. General layout of the apparatus.

IRGA, Infra-red Gas Analyser; H, Humidifier, S, Spray trap (both water-jacketed); SL, Soda lime tower; BP, By-pass; P, Pump; LC, Leaf chamber; C, Coils for temperature equalization; W, 'Wet' thermojunctions; D, 'Dry' thermojunctions; WB, Water bath controlled to give constant leaf temperature.

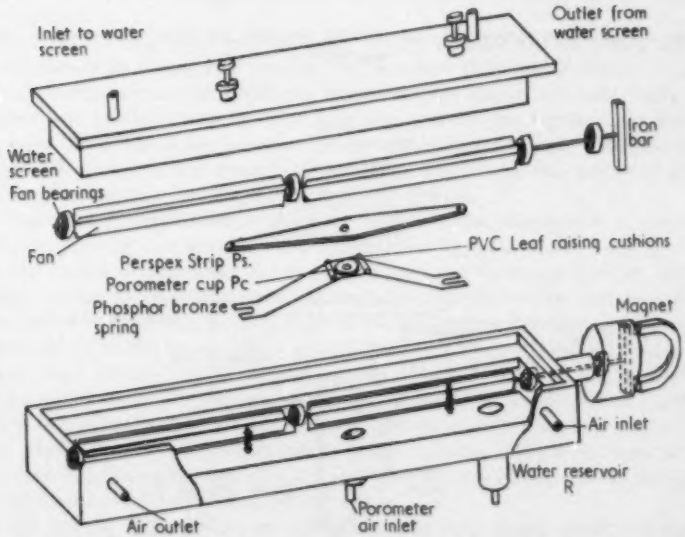

FIG. 2. Perspective view of leaf chamber and lid, also showing porometer fittings and fan separately.

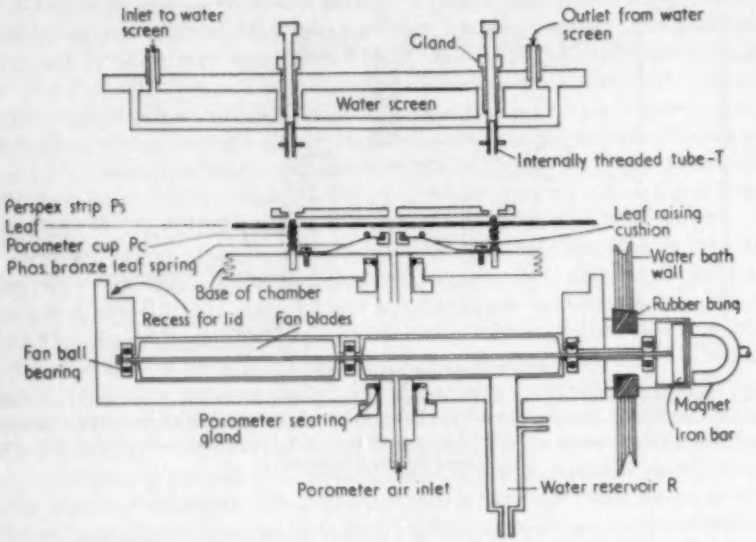

FIG. 3. Longitudinal sections of leaf chamber and lid, also showing porometer fittings separately.

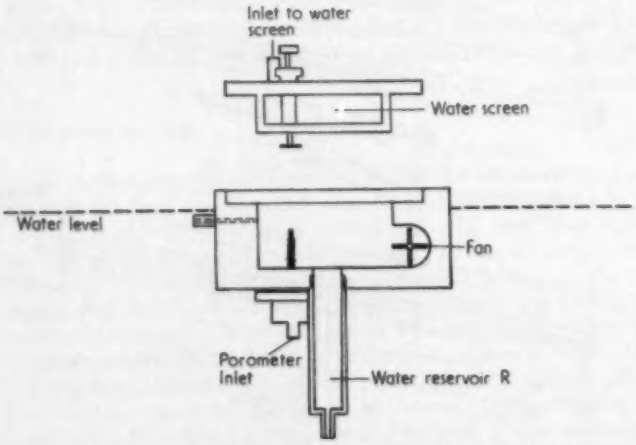

FIG. 4. Transverse section of leaf chamber and lid.

of the various wet- or dry-bulb temperatures. The two dry-junction readings (water bath temperature) and the wet-junction reading for the incoming air at 50 per cent. RH were normally constant and only required to be checked; thus only the wet-junction reading for the outgoing air was as a rule critical for measuring transpiration.

The leaf chamber. The rectangular 'Perspex' leaf chamber is shown schematically in perspective and in longitudinal and transverse section in Figs. 2, 3, and 4. The leaf was placed with its sheath in the reservoir *R*, where the solution could be changed by means of two tubes without opening the leaf chamber; its blade was supported to lie centrally along the length of the chamber and was situated between a 'Perspex' strip P_s and a spring-mounted porometer cup P_c consisting of a latex rubber grommet ring which was used without grease. In the normal position of the leaf and cup the former was supported on two 'leaf-raising cushions' of PVC tubing and air passing through the chamber could reach the leaf surfaces both within and above the cup area (Fig. 3). When a porometer reading was to be taken, P_s was screwed down by means of two internally threaded tubes *T* passing through packing glands in the chamber lid; these pressed P_c against the base of the chamber and so connected it to a Wheatstone bridge porometer (Heath and Russell, 1951). Circulation of air in the main system had to be stopped for about 2 mintues during a porometer reading. The gasometer-type aspirator for the porometer was filled with CO_2-free air, but the small volume of this injected through the leaf into the system during a reading could not be observed to affect the subsequent readings of the Infra-red Gas Analyser.

The lid of the leaf chamber was sealed on over a broad surface with a thin film of a mixture of bees-wax and 'vaseline' and was held down by G-clamps. It incorporated a water-screen with circulating water from the bath in which the body of the chamber was nearly immersed. The bath temperature was regulated to maintain a constant leaf temperature, as measured by a small thermocouple inserted into the leaf blade from below and about half-way along. No trouble was experienced with condensation droplets forming on the inside of the lid.

A four-bladed fan was fitted along the length of the chamber in a recess and driven magnetically from outside at a speed shown by a stroboscope on the motor. This was intended to circulate the air at right angles to the long axis of the leaf with strongly turbulent flow over both leaf surfaces, thus preventing the formation of appreciable CO_2 gradients perpendicular to the leaf surface. Smoke tests showed that the best speed for the fan was 200 r.p.m., when very effective mixing without stratification, dead pockets or short-circuiting was obtained. This stirring should have resulted in the air leaving the chamber with a mean CO_2 concentration practically equal to that at the leaf surface near the exit. In combination with a rate of circulation in the whole closed system (total volume about 2 litres) of about 50 litres an hour it ensured that the attainment of a steady-state CO_2 concentration was not greatly delayed by the characteristics of the apparatus.

In the present investigation, as indicated above, only closed circuit experiments were carried out, but in describing the leaf chamber for the first time it seems appropriate to mention its characteristics for use in other types of experiment also. Owing to the fact that the mass flow of air along the chamber was at right angles to the circulation due to the fan, under conditions of uptake of carbon dioxide in open circuit assimilation experiments there would be a marked concentration gradient along the leaf. This would have to be allowed for by estimating the mean effective concentra-

tion under which assimilation was occurring.[1] Even so this could be done with greater accuracy because of the stirring, which should prevent any air from passing through the chamber without coming close to the leaf surface; this can occur with stream-line flow through a deep leaf chamber, or one wider than the leaf, and may result in serious overestimates of the concentration in question (Heath, 1951). In 'semi-closed circuit' assimilation experiments (Orchard, 1956 and cf. van den Honert, 1930) rapid circulation of air in the whole system is combined with the introduction before the leaf chamber of a slow measured flow of air of known CO_2-content and the removal through an escape trap of an equal volume of air after the leaf chamber; there need then be very little concentration gradient along the chamber (or, owing to the fan, across it) and the measured concentration in the air leaving the chamber should be very close to that at the leaf surface as a whole; when the system has come to a steady state, the rate of apparent assimilation will be the difference between the rate of input of CO_2 into the system and the rate of egress with the escaping air. Since it will depend on a large difference in concentration and a slow rate of flow, both of which can be measured accurately, the errors of estimation will be low. The use of a fan should reduce the delay in attaining a steady state.

DESIGN OF EXPERIMENT AND RESULTS

Four pairs of leaves of wheat *var.* 'Charter' were used in the experiment. The first leaf of each pair was given one mannitol treatment of 0·4 molar concentration and one of 0·2 molar, the order being decided at random; the second leaf was given the two mannitol treatments in the reverse order. Each mannitol treatment was preceded and followed by a water treatment and six measurements of Γ were recorded for each leaf, i.e. before, during and after each mannitol treatment.

During the experimental period each leaf was subjected to a constant light intensity of 900 f.c. from tungsten filament lamps half immersed in running water. The leaf temperature was maintained at $25 \pm \frac{1}{2}$° C. and the relative humidity of the air entering the chamber at 50 per cent. (except as mentioned below). The leaf was first allowed to bring the CO_2 content of the circulating air to Γ. When this had been constant for at least 20 minutes it was recorded and the appropriate concentration of mannitol was substituted for the water in *R*. In every case an initial fall in CO_2 concentration was observed; this was followed by a rise, continuing generally for 1 to 2 hours, after which the concentration became almost constant (Figs. 5 and 6). There was a tendency for the concentration to creep up very slowly (see Discussion) but when it had been nearly constant for some 20 minutes the reading was taken as Γ. The mannitol solution was then replaced by water and a new end cut on the leaf sheath under water without removing it from the leaf chamber. After this the humidity in the circuit was increased almost to saturation and the water in the reservoir was renewed every 5 minutes for the first 25 minutes to remove

[1] This is usually calculated as: Mean $(CO_2) = \dfrac{C_i - C_f}{\log_e(C_i/C_f)}$, but it would be more accurate to use $\dfrac{C_i - C_f}{\log_e(C_i - \Gamma/C_f - \Gamma)}$, where C_i and C_f are the concentrations in the air entering and leaving the chamber.

mannitol diffusing out of the leaf. At the end of this period the humidity was restored to 50 per cent. RH and the CO_2 concentration was then always observed to have fallen—when it had reached a steady state and been constant for at least 20 minutes, Γ was recorded. After a further 20 minutes, Γ was again recorded to give an independent value preceding the second mannitol

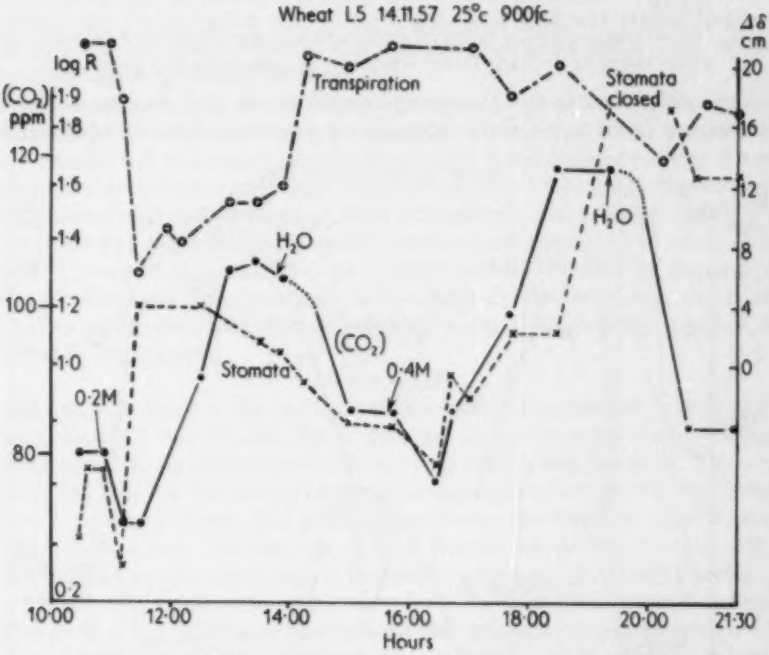

FIG. 5. Experimental records for 14.11.57 showing observed values of carbon dioxide concentration (p.p.m.), stomatal resistance (log R) and transpiration as indicated by $(W_1-D_1)-(W_2-D_2)=\Delta\delta$ cm., where W_1, W_2, D_1, and D_2 are galvanometer deflections for the wet (W) and dry (D) thermojunctions in the incoming (1) and outgoing (2) air respectively.

treatment, which was given in the same way as the first; the final water treatment also followed the routine just described.

TABLE I. *Mean* Γ *values (maintained for 20 min.), in parts per million of carbon dioxide, from eight wheat leaves*

(Leaf temperature, 25° C.; light intensity, 900 foot-candles; relative humidity, 50 per cent.)

Successive treatments	0·2 M. mannitol	0·4 M. mannitol
Water	78	77
Mannitol	94	116
Water	83	82

Significant difference for $P = 0{\cdot}05$ is 5·9; significant difference for $P = 0{\cdot}001$ is 10·6.

The mean Γ values obtained are shown in Table I and the results may be summarized as follows:

1. Each concentration of mannitol resulted in a significantly ($P < 0.001$) higher Γ value than water.
2. 0·4 M. mannitol caused a significantly ($P < 0.001$) higher Γ value than 0·2 M.
3. The average effect of order of treatment was significant at $P < 0.05$, the mean value of Γ being lower when 0·2 M. mannitol was given first.

The differences in Γ with water treatment before and after the mannitol treatments failed to reach the conventional 5 per cent. level of significance;

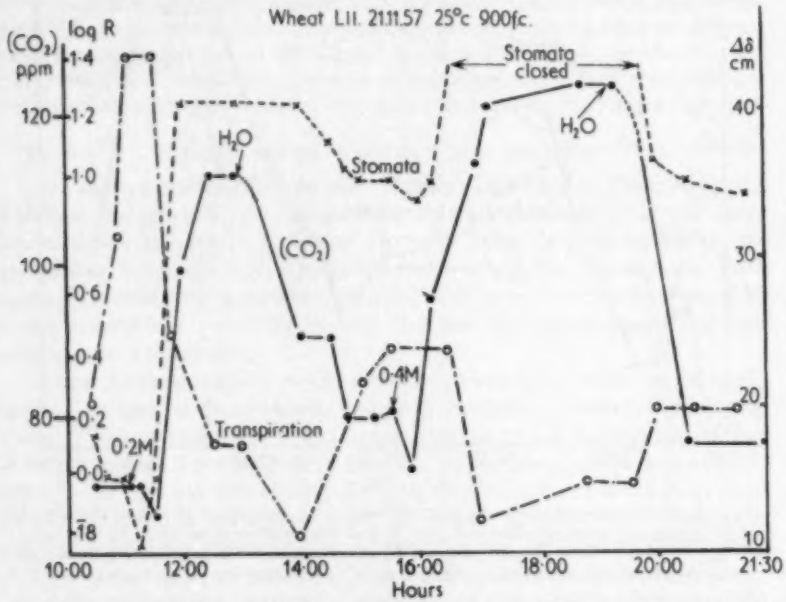

FIG. 6. Experimental records for 21.11.57 showing observed values of carbon dioxide concentration (p.p.m.), stomatal resistance (log R) and transpiration as indicated by $(W_1 - D_1) - (W_2 - D_2) = \Delta\delta$ cm., where W_1, W_2, D_1, and D_2 are galvanometer deflections for the wet (W) and dry (D) thermojunctions in the incoming (1) and outgoing (2) air respectively.

that there may have been real effects of the mannitol in increasing the Γ values subsequently measured with the leaves in water is suggested by the fact that for seven out of the eight leaves the first Γ in the morning was lower than any of the subsequent ones (see Discussion).

Concomitant observations of stomatal movement and transpiration were made, but reasonably complete records were obtained for only three leaves; Figs. 5 and 6 show the records for two of these, giving log (resistance) for the area of leaf in the porometer cup and galvanometer deflections due to the

difference in humidity of the air entering and leaving the leaf chamber. Attention may be called to the transient stomatal opening which accompanied the initial fall in carbon dioxide concentration caused by transferring the leaf sheaths to mannitol solutions; also to the stomatal closure (ultimately complete under the 0·4 M. mannitol treatment) that accompanied the subsequent rise in CO_2 and the partial reopening when the CO_2 fell again with the water treatments. Transpiration was, on the whole, correlated with stomatal movement but in the cases presented readings were not taken at the times of the transient opening referred to above.

The measured increases in Γ could have been due either to water strain caused by the osmotic effect of the mannitol in the xylem or to metabolism of mannitol if it penetrated into the living cells. A subsidiary experiment was therefore carried out, using three leaves which were subjected to water strain by removal of the water supply instead of treatment with mannitol. The CO_2 concentration rose and was allowed to do so to levels similar to the values of Γ caused by 0·4 M. mannitol; the water supply was then restored and the carbon dioxide decreased again gradually to its original level. There is therefore no need to invoke the metabolism of mannitol to explain the results obtained in the main experiment.

DISCUSSION

It has been noted in the previous section that although steady values of Γ were obtained with the leaf sheath in water, under mannitol treatment the measured CO_2 concentration tended to creep very slowly upwards. It seems likely that this was due to concentration of the mannitol solution in the xylem vessels by transpiration, thus gradually increasing the degree of water strain. The rate of change, however, was so slow that the tabulated values are probably reasonable approximations to those appropriate to 0·2 M. and 0·4 M.

Varying the temperature of the water bath (in this experiment only within a range of 1° C.) in order to maintain the leaf temperature constant at 25° C. caused small errors in the transpiration measurements, which could have been corrected by calculation had it seemed worth while; also a variation of up to ±6 per cent. in the vapour pressure deficit (VPD) of the air entering the chamber. The latter variation must have affected the degree of water strain and hence slightly the values of Γ obtained. It seemed more important, however, to avoid the effects upon Γ of a changing leaf temperature, as the water strain with a given solution in the reservoir must in any case have varied with changing stomatal aperture.

Another possible source of error is suggested by the higher values of Γ found with water after mannitol than before it, the difference being appreciable when the comparison was made with the first determination for the leaf concerned (p. 234). This might well have been due to incomplete diffusion of the mannitol out of the leaf, such diffusion being opposed by the transpiration stream. If so, a reduction of transpiration by a period of darkness in water following each mannitol treatment would have reduced the error, but the experimental design did not allow of this; as an alternative a period with

greatly increased humidity was given (p. 232) which may have been almost as effective. It is possible, however, that the higher Γ values found after mannitol treatment were not artefacts nor due to random variation but represented a persistent after-effect of partial wilting and this possibility will be investigated further. Such an after-effect would be consistent with the greater 'closing tendency' of wheat stomata after the leaf has been wilted and recovered (Milthorpe and Spencer, 1957).

The significant average effect of order of treatment, with a lower mean value of Γ when 0·2 M. mannitol was given first, might seem to suggest some sort of hardening effect. It seems, however, that it may have been fortuitous, for the initial values of Γ (with water) were lower for these leaves; this difference, which could only be due to chance, would probably tend to persist throughout the experiment.

No data were obtained in the present experiments for the changes in water content of the leaves (Milthorpe and Spencer, l.c.), which would have given a measure of the varying water strain. It can be assumed that the only appreciable resistance to water uptake was that of the living cells between the xylem vessels and the cells bordering the intercellular spaces; even with air initially of 50 per cent. RH at 23° C. (VPD 10·5 mm. Hg) passing close to the leaf surfaces it seems that, when the leaf sheath was in water, the mesophyll cells must have been nearly fully turgid with a suction pressure but little above zero. Milthorpe and Spencer suggested that in their experiments 0·2 atm. was a 'reasonable overestimate' of this suction pressure prior to wilting, and the air they passed over the leaf was dried by P_2O_5 (initial VPD 25 mm. Hg). The substitution of 0·2 M. or 0·4 M. mannitol for water is thought unlikely to have caused any plasmolysis of the leaf cells[1] and may be looked upon as roughly equivalent to increasing 'total soil moisture stress' (TSMS) from zero to 4·5 or 9·0 atm., i.e. less than one-third or two-thirds of the usually accepted permanent wilting point (15 atm.); but the resulting water strain must have been much less than this suggests owing to the absence of roots, which normally have a high resistance to water movement (Kramer, 1938).

It would thus seem that by agricultural standards, at least the milder imposed water strain was rather small; it seems at first sight remarkable that such appreciable increases in Γ were caused, that the more severe water strain was enough to cause complete stomatal closure and that the preliminary stomatal opening usually found only with severe and rapid wilting was consistently observed. The last-mentioned phenomenon will be discussed first.

Preliminary stomatal opening and fall in carbon dioxide concentration with water strain

The preliminary opening was regularly accompanied by a temporary fall of about 10 per cent. in the recorded CO_2 concentration, indicating an increase

[1] More recent tests with leaves of *var.* 'Charter' wheat (grown, however, under different conditions) show no plasmolysis of stomatal guard cells in 0·4 M. mannitol or in any concentration below 0·6 M.

in apparent assimilation rate (AA) due to the first reduction of turgor. This is somewhat reminiscent of Brilliant's (1924) finding for *Hedera helix* that AA was increased by a water deficit of 5–15 per cent. in the leaves; it was most rapid with one of 15–25 per cent. and for young leaves of *Impatiens parviflora*, with one of 25–40 per cent. More severe deficits caused a marked reduction in AA and at a certain point it fell abruptly to zero. She stated that removal of pieces of epidermis 'n'a rien changé aux resultats' so that, presumably, the stomata were not involved in any of the effects found. In the present experiment, since there was no concentration gradient across the stomatal pores at Γ immediately before applying the mannitol treatment, the stomatal opening could not have *caused* an increased rate of assimilation and hence could not be responsible for the observed fall of concentration in the system. The converse could be true and an increase in assimilation rate might have been a contributory cause of the stomatal opening observed; this seems unlikely, however, at such low levels of carbon dioxide in view of the finding (Heath and Milthorpe, 1950; Heath and Russell, 1954) that wheat stomata do not respond detectably to carbon dioxide concentration below 0·01 per cent. (It may be mentioned that a similar limit to the sensitivity of turnip stomata has recently been found by Gaastra, 1959.) If these arguments are sound, the preliminary opening and the fall in concentration were not here interdependent except in the sense that they were both caused by some common factor—ultimately, of course, the substitution of mannitol solution for water. It was noted that at these times the leaves appeared darker than usual. This suggested that possibly more light was being absorbed, which should give a higher rate of photosynthesis (though only slightly so at 900 f.c. and so low a level of CO_2) and could perhaps be due to the wrinkling of the epidermis that can occur with water loss (Fogg, 1947). The rapid loss of turgor by the epidermal cells would also, of course, on the usual theory, be responsible for 'passive' opening of the stomata. It has been pointed out by Heath and Milthorpe (1950) that the structure of the wheat leaf, with its parallel venation and large 'motor cells' in the epidermis, appears especially suitable for causing passive stomatal closure, and it might also be expected to cause transient passive opening movements. This may account for the preliminary opening occurring with degrees of water strain thought to be less severe than those, brought about by complete detachment of the leaf from a water supply, under which it has usually been observed (e.g. Darwin, 1898, Laidlaw and Knight, 1916; Scarth and Shaw, 1951; Halevy, 1956; Milthorpe and Spencer, 1957). In the present experiments the opening could scarcely have been due to the cause recently suggested by Halevy, viz. rupture of the water columns in the leaf suddenly allowing the guard cells to achieve extra turgor.

Although the temporary increase in AA that resulted from the imposition of water strain is thought unlikely to have played any part in causing the preliminary stomatal opening under the conditions of this experiment, this is not to say that it could not do so with a leaf in ordinary air of 0·03 per cent. CO_2 content. The concentration in the substomatal cavities would then

normally be much higher than Γ; an increase in AA would lower this concentration and so contribute to the opening movement. It thus seems that in wheat the preliminary opening due to water strain may be partly passive and partly active with a carbon dioxide-operated mechanism. A repetition of such experiments with other species having different leaf structure is being undertaken by one of us (H. M.) and will be described elsewhere.

Effects of water strain on apparent assimilation rate and Γ

Observations of reduction in AA with wilting have been made by many plant physiologists from the end of the last century onwards, with the picture confused, in most cases, by the probability (or certainty) of concomitant stomatal movements. Such confusion was circumvented by Scarth and Shaw (1951). They subjected detached *Pelargonium* leaves without water supply to 40–55 l./hour air flow at about 15 and 27 mm. Hg VPD in a leaf chamber and found 'true' photosynthesis (i.e. corrected for dark respiration) falling to zero in times varying, for four leaves under different conditions, from about 25 to 100 minutes from detachment. The stomata on each of the four showed preliminary opening followed by closure, and when the porometer reading had returned to the initial value (usually after about 20 minutes) the photosynthesis was always considerably lower than at the start. The fall in photosynthesis in their experiments was not, therefore, simply due to stomatal closure.

Effects of water strain in reducing AA and increasing Γ could be due to a reduced rate of photosynthesis, an increased rate of respiration, or both. Greenfield (1942) found that dark respiration of *Chlorella* was unaffected by dehydration due to immersion in sucrose-buffer solution up to 1·0 M., but photosynthesis was progressively reduced at concentrations above 0·4 M. Such reduction was almost absent with light severely limiting and was greatest at high light intensity, indicating that a dark reaction was being inhibited. This suggests that the present experiments on Γ should be repeated at various light intensities; this might not only provide evidence of inhibition of either dark reactions or the light reaction, but also test the hypothesis that the initial increase in AA with application of mannitol was due to increased light absorption.

There is evidence that water strain can increase the respiration rate of at least some higher plants. Iljin (1923) found that the respiration rate of *Ranunculus repens* rose progressively with a fall in water content, showing a 7 per cent. increase at a water deficit of 7 per cent. and a 74 per cent. increase when 36 per cent. of the water had been lost. Similar results were obtained with other species. Heath (1951) observed that passing a stream of dry air through the intercellular space system of a brightly illuminated *Pelargonium* leaf resulted in a net *output* of carbon dioxide; in similar experiments using humidified air all but an approximately constant 0·01 per cent. of CO_2 was assimilated and the dark respiration rate was reduced to about one-quarter the value.

Measurements of dark respiration were not included in the present experiment because these would still further have lengthened the experimental

periods and the completion of all six treatments in a day already necessitated 10 or 11 hours' continuous work. Also, no attempt was made to estimate the AA from the slopes of time curves for concentration (cf. Decker, 1959*a*, *b*) as such closed system experiments are considered to be appropriate only for the determination of steady-state values (Γ). The slope of the curve at any instant is a function not only of the rates of CO_2 uptake and emission by the chloroplasts and mitochondria respectively and of the external concentration at the instant concerned, but of the preceding changes in that concentration with time and the various (unknown) resistances to CO_2 movement; there is further the likelihood of systematic errors due to CO_2 being given out or absorbed by components of the apparatus (especially the humidifiers) with changing concentration in the system. For these reasons AA should be determined from 'open circuit' or 'semi-closed circuit' experiments in which a constant renewal of the CO_2 supply makes it possible to maintain a steady state during photosynthesis (p. 232). Similar considerations will apply to the estimation in a closed system of dark respiration, if this is affected by external CO_2 concentration, or if the changes of the latter cause rapid changes of stomatal aperture.

Water strain and stomatal closure

In the present work, as mentioned, no information was obtained as to the relative roles of changes in photosynthesis and respiration rates in bringing about the observed effects of water strain on Γ, but the effects in themselves are of some interest (p. 240) and especially so in relation to stomatal behaviour. As already noted (p. 228) wheat stomata were found to show closing responses to moving dry air by Heath and Milthorpe (1950), who thought that these might perhaps be brought about by loss of leaf turgor causing shrinkage of the lamina at right angles to the long axes of the stomata. The effects they observed were unlikely to have been produced by CO_2 changes, for the experiment in question was carried out with CO_2-free air and hence the concentration in the substomatal cavities was probably kept below 0·01 per cent. Again, in the present experiment, the closures actually found seem unlikely to have been due mainly, if at all, to the observed increases in CO_2. Thus Heath and Russell (1954) found maximal opening of wheat stomata (under 800 f.c. illumination) with air of 0·017 per cent. CO_2 content and 50 per cent. or 75 per cent. RH at 28° C. forced through the leaf; even with 0·029 per cent. CO_2 the closure was not great. In the present experiment, on the other hand, 'complete' closure, i.e. to beyond the sensitivity of the Wheatstone bridge porometer (cf. Heath and Russell, 1951), was obtained with a substomatal cavity CO_2-concentration (Γ) of about 0·012 per cent. (Figs. 5 and 6). Unless, therefore, loss of turgor causes a very greatly increased sensitivity to CO_2 (and there is in fact some evidence of this in work now in progress), it would seem that in wheat a large part, and probably the main part, of the closing response even to mild water strain is due to a mechanism other than that operating via increases of the CO_2 concentration in the substomatal cavities. When,

however, wheat leaves are subjected to water strain in ordinary air, so that this concentration increases from a level considerably above Γ, the CO_2-operated mechanism may be expected to contribute to the closure.

Scarth and Shaw (1951) found with *Pelargonium* that under their wilting conditions (p. 238) the preliminary stomatal opening period was prolonged to about 40 minutes by the use of CO_2-free air, both for a leaf in light and for one in the dark. This implied that the stomata were sensitive to CO_2 under such water strain and hence that the depression of photosynthesis found with the leaves in ordinary air must have been in part responsible for the closure then observed.

Carbon dioxide gradients into the leaf

Finally it may be noted that the increases in Γ of about 16 and 39 p.p.m., caused by the two mannitol treatments (Table I), represent reductions of 7 per cent. and 18 per cent. respectively in the maximum possible potential for CO_2-diffusion from ordinary air into the leaf. Such a maximum potential can, of course, only obtain with the stomata completely closed and with open stomata the intercellular space CO_2 will always be higher than Γ. Values of Γ, however, give a good (inverse) measure of the efficiency for net CO_2-absorption under the given conditions of temperature and water strain,[1] with the stomatal factor eliminated and CO_2 'limiting'. The meaning here attached to CO_2 limitation is that used by Maskell (1928), i.e., conditions such that a small increase in concentration causes an approximately proportionate increase in AA. As he points out, 'For land leaves this appears . . . to hold over a range of CO_2 concentrations very much exceeding that of the normal atmosphere, and apparently up to an assimilation rate approaching the temperature or light limit.' Such CO_2 'limitation' does not mean that *no* increase in AA can be caused by an increase in light or temperature. The practical importance of the present results is that they suggest that even relatively mild water strain may be of importance in reducing dry-matter production by the wheat plant, both by reducing the potential for CO_2-diffusion and even more by increasing the (stomatal) resistance.

ACKNOWLEDGEMENTS

Thanks are due to Mr. F. S. Smith for the skill and patience with which he constructed and modified the leaf chamber; also to the University of Natal for giving H. Meidner leave of absence and the Nuffield Foundation for supporting him with a research grant. The experimental results presented form part of a thesis for which the Ph.D. degree of the University of London was awarded.

LITERATURE CITED

BRILLIANT, B. (1924). Le Teneur en eau dans les feuilles et l'energie assimilatrice. *Compte rendus des Sc. Acad. Sci. Paris*, **178**, 2122–5.

[1] Light intensity has little effect on Γ except at low levels which, however, rise with temperature (Orchard, 1956).

DARWIN, F. (1898). Observations on stomata. *Phil. Trans. Roy. Soc. B.* **190,** 531–621.

DECKER, J. P. (1959*a*). Comparative responses of carbon dioxide outburst and uptake in tobacco. *Plant Physiol.* **34,** 100–2.

—— (1959*b*). Some effects of temperature and carbon dioxide concentration on photosynthesis of Mimulus. Ibid. **34,** 103–6.

EGLE, K., and SCHENK, W. (1953). Der Einfluss der Temperatur auf die Lage des CO_2-Kompensationspunktes. *Planta,* **43,** 83–97.

FOGG, G. E. (1947). Quantitative studies on the wetting of leaves by water. *Proc. Roy. Soc. B.* **134,** 503–22.

GAASTRA, P. (1959). Photosynthesis of crop plants as influenced by light, carbon dioxide, temperature, and stomatal diffusion resistance. *Mededel. Landbouwhogesch. Wageningen,* **59,** 1–68.

GABRIELSEN, E. K. (1948). Threshold value of carbon dioxide concentration in photosynthesis of foliage leaves. *Nature,* **161,** 138.

GLOVER, J. (1941). A method for the continuous measurement of transpiration of single leaves under natural conditions. *Ann. Bot.* N.S. **5,** 25.

GREENFIELD, S. S. (1942). Inhibitory effects of inorganic compounds on photosynthesis in *Chlorella. Amer. J. Bot.* **29,** 121–31.

HALEVY, A. (1956). Orange leaf transpiration under orchard conditions. IV. A contribution to the methodology of transpiration measurements in Citrus leaves. *Bull. Res. Counc. Israel,* **5D,** 155–64.

HEATH, O. V. S. (1939). Experimental studies of the relation between carbon assimilation and stomatal movement. I. Apparatus and technique. *Ann. Bot.* N.S. **3,** 469–95.

—— (1941). Experimental studies of the relation between carbon assimilation and stomatal movement. II. The use of the resistance porometer in estimating stomatal aperture and diffusive resistance. Part 1. A critical study of the resistance porometer. Ibid. N.S. **5,** 455–500.

—— (1949). Carbon dioxide in the intercellular spaces of leaves during photosynthesis. *Nature,* **164,** 822.

—— (1950). Studies in stomatal behaviour. V. The rôle of carbon dioxide in the light response of stomata. Part 1. Investigation of the cause of abnormally wide stomatal opening within porometer cups. *J. Exp. Bot.* **1,** 29–62.

—— (1951). Assimilation by green leaves with stomatal control eliminated. *Symp. Soc. Exp. Biol.* **5,** 94–114.

—— and MEIDNER, H. (1958). Effects of water-strain on the minimum intercellular space carbon dioxide concentration Γ in wheat leaves. *Nature,* **182,** 1524–5.

—— and MILTHORPE, F. L. (1950). Studies in stomatal behaviour. V. The rôle of carbon dioxide in the light response of stomata. Part 2. Preliminary experiments on the interrelations of light intensity, carbon dioxide concentration and rate of air flow in controlling the movement of wheat stomata. *J. Exp. Bot.* **1,** 227–43.

—— and ORCHARD, B. (1957). Midday closure of stomata. Temperature effects on the minimum intercellular space carbon dioxide concentration 'Γ'. *Nature,* **180,** 180–1.

—— and RUSSELL, J. (1951). The Wheatstone Bridge Porometer. *J. Exp. Bot.* **2,** 111–16.

—— —— (1954). Studies in stomatal behaviour. VI. An investigation of the light responses of wheat stomata with the attempted elimination of control by the mesophyll. Part 2. Interactions with external carbon dioxide, and general discussion. Ibid. **5,** 269–92.

VAN DEN HONERT, T. H. (1930). Carbon dioxide assimilation and limiting factors. *Rec. Trav. Bot. Neerlandais,* **27,** 149–286.

ILJIN, W. S. (1923). Einfluss des Welkens auf die Atmung der Pflanzen. *Flora* N.F. **16,** 379–403.

KRAMER, P. J. (1938). Root resistance as a source of the absorption lag. *Amer. J. Bot.* **25,** 110–13.

LAIDLAW, C. G. P., and KNIGHT, R. C. (1916). A description of a recording porometer and a note on stomatal behaviour during wilting. *Ann. Bot.* **30,** 47–56.

MASKELL, E. J. (1928). Experimental researches on vegetable assimilation. XVIII. The relation between stomatal opening and assimilation. A critical study of assimilation rates and porometer rates in leaves of cherry laurel. *Proc. Roy. Soc. B.* **102,** 488–533.

MEIDNER, H. (1958). *Studies of diffusion phenomena in leaves.* Ph.D. Thesis, University of London.

—— and HEATH, O. V. S. (1959). Studies in stomatal behaviour. VIII. Stomatal responses

to temperature and carbon dioxide concentration in *Allium cepa* L. and their relevance to midday closure. *J. Exp. Bot.* **10**, 206–19.

MILLER, E. S., and BURR, G. O. (1935). Carbon dioxide balance at high light intensities. *Plant Physiol.* **10**, 93–114.

MILTHORPE, F. L., and SPENCER, E. J. (1957). Experimental studies of the factors controlling transpiration. III. The interrelations between transpiration rate, stomatal movement and leaf water content. *J. Exp. Bot.* **8**, 414–37.

ORCHARD, B. (1956). *Studies in carbon assimilation.* Ph.D. Thesis, University of London.

RABINOWITCH, E. I. (1951, 1956). Photosynthesis and related processes. Vol. II, parts 1 and 2. Interscience Publishers, New York.

SCARTH, G. W., and SHAW, M. (1951). Stomatal movement and photosynthesis in *Pelargonium.* II. Effects of water deficit and of chloroform: photosynthesis in guard cells. *Plant Physiol.* **26**, 581–97.

Gas Exchange between *Equisetum limosum* and its Environment[1]

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WITH TWO FIGURES IN THE TEXT

Received 14 September 1960

SUMMARY

Equisetum limosum grows in standing water. Its perennating rhizomes ramify in the bottom mud, which is a markedly oxygen-deficient medium. During the summer months aerial shoots are present and extend above the surface of the water to a height of about 1 m. The concentration of oxygen in the internal atmosphere of plants in the field decreases progressively from the stem to the perennating rhizomes. There is a reverse gradient of carbon dioxide concentrations.

The structure of the nodal diaphragms of the aerial stem is described and the progressive occlusion of the pores of the diaphragms between May and November is demonstrated. This occlusion of the diaphragms has been correlated with a reduction in the ease of gaseous diffusion along lengths of aerial stems.

Oxygen is present in the rhizomes throughout the winter, despite the fact that the dead aerial stems have broken off and the stumps have been submerged by the rise in water level, thus isolating the rhizome from atmospheric oxygen.

The results are discussed in relation to the ability of the plant to grow in an oxygen-deficient environment.

INTRODUCTION

WHEN plants grow in standing water their roots are in a medium which is normally deficient in oxygen. Such plants generally exhibit extensive development of aerenchyma, and it has usually been considered that the oxygen requirements of the roots are met by the diffusion of oxygen through this tissue from the shoot. It has, for example, been shown by Conway (1937) that oxygen is present at near-atmospheric concentrations in the roots of *Cladium mariscus* growing in the water-logged conditions of its natural habitat, and that there was a continuity of the air spaces of the plant from the leaves to the roots; it was thus demonstrated that the diffusion of gas is possible through the plant, but the method employed did not allow it to be proved that the oxygen requirement of the roots was in fact supplied in this way. The present work was initiated with the object of providing more direct evidence on this question, using a different plant species.

Equisetum limosum was considered to be a suitable plant for this investigation since it grows in standing water, with its perennating organs ramifying in an oxygen-deficient medium. Moreover, the small number of physiological

[1] The material of this paper formed part of a thesis presented to the University of Liverpool for the degree of Ph.D.

investigations which have hitherto been carried out with vascular cryptogams was a further consideration influencing its choice. The horizontal rhizome, which branches freely in the underlying mud, is usually 8–20 mm. in diameter and gives rise at intervals to erect rhizomes some 6–12 mm. in diameter, the ends of which elongate to form aerial stems 2–8 mm. in diameter. The horizontal rhizomes are perennial; the bases of the erect rhizomes appear to be usually biennial, and the aerial stems are annual. These emerge from the water in late April, grow to a height of up to 1 m. and die off in early November. Buds, which elongate in the following season to form new aerial stems, are developed on the erect rhizomes some 15–20 cm. below the water level. Adventitious roots are developed freely at the nodes of the horizontal rhizomes and to a lesser extent of the erect rhizomes. Leaves, reduced to fused sheaths at the nodes, are present throughout the plant.

The rhizomes and the aerial stems are hollow, the central canal being interrupted only by the nodal diaphragms. This canal occupies about four-fifths of the diameter of the stem, whereas in the rhizomes, which have in addition a ring of small peripheral (vallecular) canals, the central canal occupies only about half the diameter. As the structure of the diaphragms was of obvious importance in the study of gas exchange, it was investigated. Early in the season the diaphragms of the aerial stem consist of layers of thin-walled cells between which there are pores; as the season advances the cell walls thicken and the pores become progressively occluded. The reduction in the pores is particularly strikingly shown in the transverse sections of the diaphragms (Fig. 1 A and B). These observations encourage inquiry as to whether the rate of diffusion decreases as the season advances. In the submerged rhizome by contrast the diaphragms remain relatively unthickened except when rhizomes have been injured. It was observed that damaged parts of the plant can be effectively sealed off by the occlusion of the diaphragms.

The material used in the investigation grew in a dense, almost exclusive stand in a pond with a muddy bottom containing a high proportion of sand overlying a heavy clay substratum. There was abundant decomposing matter at the surface of the mud and the water was slightly turbid. The growth of *E. limosum* was confined to areas where the depth of water did not exceed 1·5 m. During the summer, water samples were analysed by the micro-Winkler method (Fox and Wingfield, 1938). The oxygen content fell off rapidly from the surface. At 30 cm. the oxygen content was 3·4 ml. per litre, i.e. about half that of water in equilibrium with air. At a depth of 50 cm. and greater the oxygen content was below the limits of detection (about 1 ml. per litre).

EXPERIMENTAL

1. *Diffusion gradients in plants growing in the field*

Samples of gas were extracted from the air-space systems of plants growing in the field by means of a portable vacuum-extraction apparatus (Vallance and Coult, 1951). The plants were divided into the following regions: (*a*) aerial

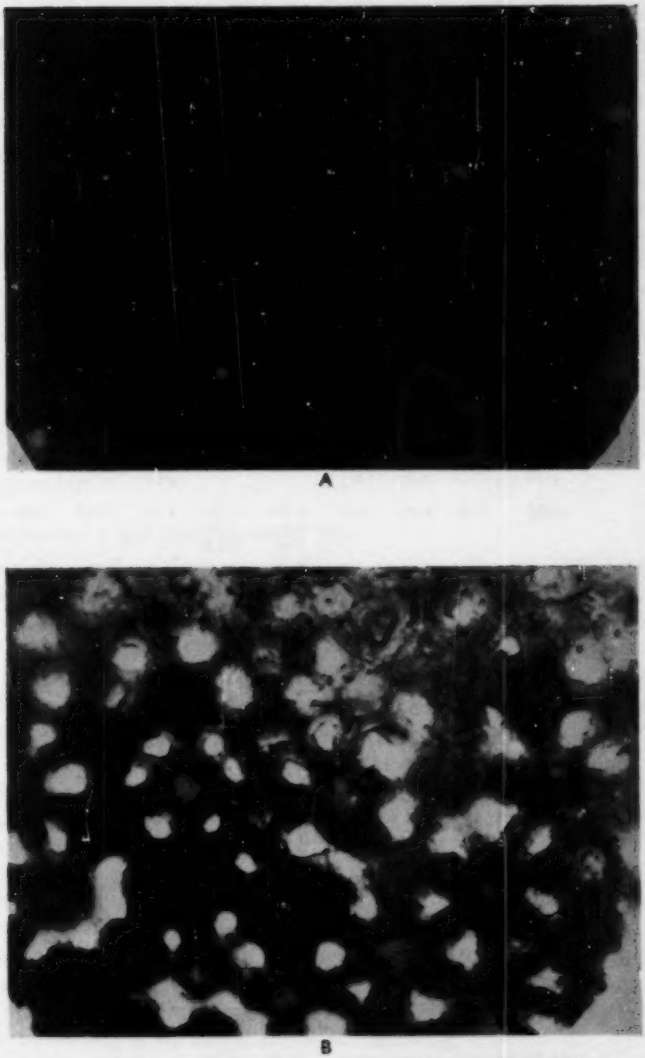

FIG. 1. Photomicrographs of transverse sections of the diaphragms of the aerial stem of *E. limosum*, illustrating the occlusion of the diaphragm pores as the season progresses.

A. section prepared in August.
B. section prepared in November.

stem, (*b*) water level to bud, (*c*) bud, (*d*) bud to 15 cm. along the erect rhizome, (*e*) horizontal rhizome. Extraction from all regions was completed within 10–15 minutes of harvesting the plants so that errors due to shock-induced respiration should have been kept to a minimum (Laing, 1940). The samples were taken to the laboratory and analysed for oxygen and carbon dioxide in the micro-gas-analysis apparatus described by Vallance and Coult (1951)

The results of analyses carried out on various dates are given in Table I. Considerable variations occurred, as is to be expected in field data, but certain

TABLE I

The composition of the internal atmospheres of different regions of plants of Equisetum limosum, *growing in the field*

Month	Aerial stem		Water L.-bud		Bud		Bud-15 cm.		Horizontal rhizome	
	CO_2%	O_2%	CO_2%	O_2%	CO_2%	O_2%	CO_2%	O_2%	CO_2%	O_2%
Aerial stems present										
June	0·60	19·6	2·93	12·8			3·17	11·5		
	0·45	19·7	1·67	18·9			3·50	14·8		
	1·166	18·8	2·38	16·1			4·65	12·5		
	1·14	19·2	2·60	15·1			4·96	12·1		
	1·18	19·2	2·07	16·3			4·07	12·8		
August	0·68	19·2	2·94	14·0	6·62	5·60	3·25	7·98	5·05	3·54
					7·73	5·85				
					5·77	6·68				
					5·77	7·70				
					6·91	4·40				
	0·0	20·4	3·08	14·6	5·10	10·5	—	—	5·05	10·7
	0·59	19·9	4·07	13·7	4·40	14·3	5·37	8·08	6·40	6·85
	3·10	18·0	6·70	7·33	3·25	7·40	8·00	8·55	8·60	8·35
September	2·66	15·1	2·98	4·17	5·58	4·27	5·75	6·96	4·73	5·62
	0·0	18·7	2·15	15·7	6·76	3·75	6·14	6·90	5·45	7·89
	0·84	19·6	3·63	7·3			4·27	5·75	5·30	9·90
October	0·29	19·0	1·93	14·3	2·56	10·9	1·96	10·4	4·85	7·60
	1·20	15·3	3·05	8·43	4·05	5·79	5·20	3·13	4·75	2·36
	0·28	18·8	3·36	1·04	3·81	11·4	4·63	4·18	—	—
Aerial stems absent										
December					3·88	4·16				
					2·55	1·13				
					2·65	0·43				
					3·88	5·40				
					0·87	2·90				
January					4·34	3·47				
					3·45	0·52				
					4·33	1·90				
April	(*a*) Buds still dormant								0·0	1·3
									1·96	0·3
									0·24	2·6
	(*b*) Buds begin to elongate				3·17	2·60			1·65	4·4
					0·84	4·50			2·05	5·0
					2·53	1·13			1·13	7·6
					3·36	5·90			0·56	5·5
	(*c*) Buds about 1–2 cm. above water level				1·1	5·8			0·56	11·8
					0·85	4·85			0·85	8·0
					1·07	2·95			3·0	6·7

broad trends are apparent. Between May and October, when the aerial stems were present, their oxygen content was close to that of the atmosphere and considerably higher than that of the horizontal rhizomes. Conversely, the carbon dioxide concentration was greater in the horizontal rhizome than in the aerial stems. The values for gas in the intermediate tissues fell between these limits. There is thus clear evidence of an oxygen gradient from the aerial to the submerged parts of the plants and a reverse gradient of carbon dioxide. Samples taken between November and May when no aerial stems were present again showed appreciable concentrations of oxygen in the horizontal rhizomes. The values returned approximately to the summer levels when the emerging stems were 1–2 cm. above the water level. The comparison of values for buds with adjacent rhizome tissue shows appreciable variation in gas content, sometimes by factors exceeding 2. It is presumed that these reflect differences in the metabolic rate of the buds and of ease of diffusion into them.

In view of the high solubility of carbon dioxide in water, the high concentrations of this gas in the horizontal rhizomes suggests that the outer layers of the tissue are relatively impermeable to the passage of gas. The oxygen content of the rhizomes, especially during the winter months, is strong evidence in the same direction.

2. *The passage of gas along an aerial stem*

The passage of gas along an aerial stem was investigated in the laboratory. For this purpose a length of stem 40 cm. long was cut and its cut ends sealed with mercury retained by lanolin plugs. The stem was then sealed at its midpoint into a glass tube 2 cm. long and the space between the stem and the glass filled with mercury held in place with lanolin. This tube was in turn sealed with luting wax (90 per cent. beeswax and 10 per cent. 'Vaseline') into a ground-glass cone, either side of which was connected to a large Barcroft respirometer. By manipulating the two respirometers in the normal manner it was possible to examine the transfer of gas along the stem. Except for its smaller size, the apparatus was identical with that described by Coult and Vallance (1958).

Normally the respirometer attached to the lower part of the stem was filled initially with nitrogen, while the upper chamber was filled with air or a mixture of 20 per cent. oxygen and either 3 or 12 per cent. carbon dioxide made up to 100 per cent. with nitrogen. Three consecutive runs of 21 hours duration were carried out on each stem and the results discarded if, at the end of this time (4 days), the stem was found to be damaged either by rupture of the tissue, by wilting or by fungal attack or yellowing.

The results of six experiments are presented in Table II. In most cases oxygen diffused along the stem and was present in measurable amounts in the lower chamber. The mean R.Q.s for all experiments fell between 0·84 and 1·41. Thus the respiration appeared to have been largely aerobic, indicating that sufficient oxygen had diffused through the stem to meet the respiratory

Table II

The movement of gas along an aerial stem of E. limosum *as determined by the change in the composition of the atmosphere of two Barcroft respirometer chambers over a 21-hour period*

For details, see text.

	Oxygen				Carbon Dioxide					Nitrogen			
	Initial	Final		Loss	Initial	Final		Gain		Upper chamber		Lower chamber	
Date		Upper	Lower			Upper	Lower		R.Q.	Initial	Final	Initial	Final
(i) Upper chamber containing *air*													
June 1*	13·43	8·37	2·37	2·69	0·0	1·46	1·27	2·73	1·02	48·82	51·81	76·30	73·11
2	13·50	7·82	2·28	3·40	0·0	1·36	1·02	2·38	0·70	49·10	52·77	76·26	72·11
3	13·50	9·90	1·16	2·44	0·0	0·70	1·28	1·98	0·81	49·05	51·58	76·40	73·55
Mean	13·48	8·69	1·90	2·84	0·0	1·17	1·19	2·36	0·84	48·99	52·05	76·32	72·92
September 1	14·55	11·67	0·71	2·17	0·0	1·45	1·73	3·18	1·46	53·30	54·78	77·73	76·09
2	14·60	11·90	0·89	1·81	0·0	1·40	0·76	2·16	1·20	53·30	54·40	77·83	76·70
3	14·60	11·70	0·36	2·54	0·0	1·15	0·75	1·90	0·75	53·40	54·95	77·60	76·69
Mean	14·58	11·76	0·65	2·17	0·0	1·33	0·75	2·41	1·13	53·33	54·71	77·72	76·49
(ii) Upper chamber containing 3% CO_2 + 20% O_2													
June 1	12·27	9·28	2·41	0·58	1·90	1·98	1·27	1·35	2·32	48·10	51·15	78·10	75·38
2	12·30	9·20	1·78	1·32	1·90	1·88	1·23	1·31	1·00	48·31	51·45	78·50	75·74
3	12·30	9·15	1·08	2·07	1·88	2·04	1·71	1·87	0·90	48·22	50·95	78·45	75·87
Mean	12·29	9·21	1·76	1·66	1·89	1·97	1·40	1·51	1·41	48·21	51·18	78·35	75·66
July 1	13·90	11·85	1·18	0·87	2·27	2·50	1·25	1·48	1·70	54·37	57·86	75·62	72·50
2	14·00	9·60	1·06	3·34	2·29	2·90	1·84	2·45	0·73	54·73	58·42	75·40	72·20
3	13·90	10·20	1·58	2·12	2·28	2·70	1·75	2·17	1·02	54·64	57·92	75·70	72·38
Mean	13·93	10·55	1·27	2·11	2·28	2·70	1·61	2·03	1·15	54·58	58·06	75·57	72·36
(iii) Upper chamber containing 12% CO_2 + 20% O_2													
May 1	12·85	10·20	2·08	0·57	7·72	6·52	1·84	0·64	1·12	43·68	47·78	77·45	73·58
2	12·87	9·69	0·0	3·18	7·72	7·40	3·27	2·95	0·93	43·71	47·31	77·80	74·63
3	12·90	10·47	0·45	1·98	7·74	8·39	1·07	1·72	0·87	44·01	45·69	77·65	76·37
Mean	12·87	10·12	1·15	1·91	7·73	7·44	2·06	1·77	0·97	43·80	46·93	77·63	74·84
September 1	14·50	13·37	0·0	1·13	8·22	7·50	1·72	1·00	0·91	47·68	48·52	76·17	75·13
2	14·60	12·90	0·80	0·90	8·26	7·60	1·61	0·95	1·05	47·94	49·30	76·60	75·29
3	14·60	12·40	0·0	2·20	8·25	8·12	1·81	1·68	0·76	47·75	49·52	76·50	74·62
Mean	14·53	12·89	0·27	1·41	8·24	7·74	1·71	1·23	0·91	47·79	49·11	76·42	75·01

* 1, 2, and 3 represent the results for the three consecutive 21 hr. periods.

requirements of the lower half. When a marked carbon dioxide gradient existed at the commencement of the experiment, this gas also diffused through the stem into the lower chamber.

The utilization of oxygen and the production of carbon dioxide in respiration influenced the observed movement of both gases between the chambers.

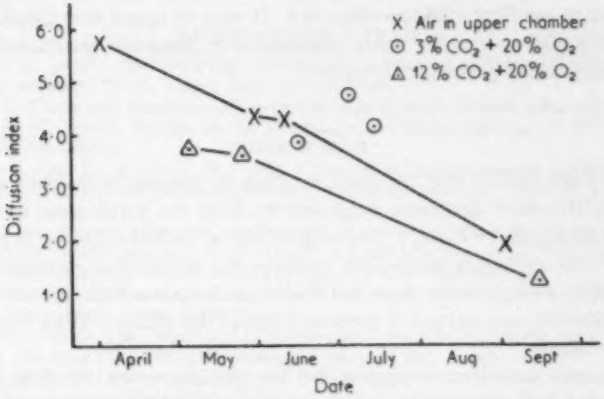

FIG. 2. The decrease in the rate of diffusion of nitrogen along a length of aerial stem of *E. limosum* between April and September.

However, the movement of nitrogen will have been unaffected. An examination of the rate at which it diffused is thus of particular interest. The rate of diffusion along different stems can be conveniently compared by dividing the volume of nitrogen that diffused through the plant by the volume that would have diffused from the lower to the upper chamber if no barrier existed between the two chambers. This value, defined as the 'diffusion index', was determined at various times in the season with either air or a mixture of 12 per cent. carbon dioxide and 20 per cent. oxygen in the upper chamber (Fig. 2). The values of the indices ranged from 1–6, decreasing during the season. Determinations over a more limited period were made in 3 per cent. carbon dioxide; these fell in the same general range.

3. *Entry of gas into the aerial stem*

The passage of gas along the stem in the experiments described above could have been limited either by the rate of diffusion across the diaphragms or by the entry of gas across the epidermis and cortex. An attempt was therefore made to investigate the reactions of the stomata of the aerial stem to various gaseous conditions using the injection technique of Molisch (1912) as modified by Williams (1949). Stems were placed either in an atmosphere devoid of carbon dioxide or in a mixture of 5 per cent. carbon dioxide in air and illuminated for one hour. They were then plunged into an 0·05 per cent.

solution of Gentian violet in absolute alcohol. After rinsing, it was seen that the stomatal cavities of the stems which had been in the CO_2-free atmosphere had been injected with the dye; those that had been exposed to CO_2 did not show this injection. Qualitative evidence was obtained, therefore, that stomatal movement takes place. Porometer experiments, however, indicated that under all conditions gas moved so freely across the stem that no change in the resistance to gas flow could be observed. It may be noted that Dosdall (1919) in an investigation into the water relations of *E. fluviatilis* concluded that the stomata remained permanently open.

DISCUSSION

It has been shown that the concentration of oxygen in the internal atmosphere of the plant decreases progressively from the aerial stem to the horizontal rhizome, there being a reverse gradient of carbon dioxide. It is evident that little gas exchange takes place between the submerged rhizomes and the surrounding environment since the rhizomes contain a high concentration of carbon dioxide and oxygen is present even in the winter, when they are cut off from the air.

Laboratory experiments suggest that the rate of gaseous diffusion down the aerial stems decreases as the season progresses. There is also evidence that the stomatal control in the stem is small since the stomata always appeared to be open. None the less it is of interest that stomatal movement in response to the presence of carbon dioxide was demonstrated by the injection technique and Fig. 2 suggests that in a carbon dioxide concentration of 12 per cent. the rate of movement of gas along the stem is reduced. This cannot, however, be considered as conclusive evidence of stomatal movement since the differences are small; moreover, it is possible that high concentrations of carbon dioxide may have had other effects.

The evidence presented is compatible with the view that the rate of diffusion down the aerial stem is governed by the porosity of the diaphragms; when the diaphragms were occluded the rate of diffusion went down.

It is concluded that the oxygen requirements of the plant throughout the summer are provided by diffusion from the aerial stem. In the overwintering period there is evidence of the storage of oxygen. In this connexion it is of interest that rhizomes can exist for moderate periods in low oxygen concentrations and in nitrogen (Barber, 1956). It has also been shown that in low oxygen concentrations the rhizomes can fix carbon dioxide into organic acids and that lactic acid and not alcohol is produced under anaerobic and near-anaerobic conditions (Barber, 1957).

It may be considered therefore that the ability of the rhizomes of *E. limosum* to grow in an oxygen-deficient environment reflects both (i) an efficient system for oxygen diffusion from the aerial stems in summer, and (ii) a facility for oxygen storage, particularly in winter, and biochemical adaptation to conditions of low oxygen supply.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge my indebtedness to my supervisor, Mr. D. A. Coult, and to Professor N. A. Burgess for their interest in this work and to the D.S.I.R. for financial assistance. I am grateful to Dr. R. Scott Russell for his helpful criticism of this manuscript.

LITERATURE CITED

BARBER, D. A. (1956). *The Physiology of* Equisetum limosum *with special reference to its gaseous exchange.* Ph.D. Thesis, Univ. of Liverpool.

—— (1957). Lactic acid formation and carbon dioxide fixation. *Nature*, **180**, 1053.

CONWAY, V. M. (1937). Studies in the autecology of *Cladium mariscus R. Br.* Part III. *New Phytol.* **36**, 64.

COULT, D. A., and VALLANCE, K. B. (1958). Observations on the gaseous exchanges which take place between *Menyanthes trifoliata L.* and its environment. Part II. *J. Exp. Bot.* **9**, 384.

DOSDALL, L. M. (1919). The water requirements of *Equisetum*. *Plant World*, **22**, 1.

FOX, H. MUNRO, and WINGFIELD, C. A. (1938). A portable apparatus for the determination of oxygen dissolved in a small volume of water. *J. Exp. Biol.* **15**, 437.

LAING, H. E. (1940). The composition of the internal atmosphere of *Nuphar advenum* and other water plants. *Amer. J. Bot.* **27**, 861.

MOLISCH, H. (1912). Das Offen- und Geschlossensein der Spaltöffnungen, veranschaulicht durch eine neue Methode (Infiltrationsmethode). *Z. Bot.* **4**, 106.

VALLANCE, K. B., and COULT, D. A. (1951). Observations on the gaseous exchanges which take place between *Menyanthes trifoliata L.* and its environment. Part I. *J. Exp. Bot.* **2**, 212.

WILLIAMS, W. T. (1949). Studies in stomatal behaviour, III. The sensitivity of stomata to mechanical shock. *Ann. Bot.*, N.S. **13**, 309.

The Relationship between Metabolism and the 'Exchangeability' of Ions in Plant Tissues

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WITH TWO FIGURES IN THE TEXT

Received 25 August 1960

SUMMARY

The uptake of the rubidium ion by well-washed disks of carrot parenchyma has been determined at 0·2° C and 25° C., in the presence and absence of 10^{-4}M. dinitrophenol, from solutions of rubidium chloride containing 0·5 and 5·0 meq./l. Readily-exchangeable and non-exchangeable fractions were separately indentified.

The lowering of temperature and application of DNP reduced the magnitude of both ion fractions at each of the two concentrations of rubidium chloride. Despite the fact that the uptake of ions into an exchangeable form at 5·0 meq./l. was about 3 times greater, the combined effect of low temperature and the presence of DNP reduced this fraction by a relatively constant absolute amount. Under the same conditions the uptake of ions into a non-exchangeable form from each concentration was reduced by approximately the same percentage.

Over a 6-hour period the rate of uptake of rubidium into a non-exchangeable form at 25° C. was relatively constant, whereas at 0·2° C. there was an initial rapid uptake lasting for about 60 minutes followed by a slow steady uptake. The Q_{10} of this latter process measured after 360 minutes was 2·3.

It is concluded that an appreciable part of the capacity of the tissue to hold ions by exchange is dependent on concurrent metabolism.

The significance of measurements of exchangeability in the interpretation of mechanisms of ion uptake is discussed.

INTRODUCTION

In many investigations of nutrient absorption by plant tissues the ability of ions to exchange with those in the external medium has been regarded as a criterion for determining the nature of the mechanism by which they have been absorbed. Purely physical processes are generally regarded as responsible for the entry of ions which remain readily exchangeable, and the initial rapid flux of absorption, which is particularly marked with cations, is widely explained in these terms. Conversely the retention of ions in non-exchangeable forms has been taken as evidence of their absorption by metabolic processes. Accordingly, in studies of the relationship between metabolism and the active transport of ions in plant tissues, consideration has sometimes been given only to that fraction of the entering ions which is non-exchangeable (Epstein, 1956; Fried and Noggle, 1958). Similarly, it has been assumed that the quantity of ions which will normally enter tissues in an exchangeable form is little changed when the temperature is lowered to near 0° C, although metabolism is greatly reduced (Briggs *et al.*, 1958; Sutcliffe, 1954). Some suggestion that this

analysis is inadequate has, however, been provided by indications that the quantity of ions held in tissues in an exchangeable form may be reduced both by the presence of respiratory inhibitors and by low temperatures (Russell and Ayland, 1955; Ayland, 1956). The present study was undertaken to obtain more critical information on this question.

EXPERIMENTAL METHODS

Disks of phloem parenchyma of carrots, 8 mm. in diameter and 1 mm. thick, were washed for 6 days in glass-distilled water, which was changed twice daily. The absorption of the rubidium ion labelled with ^{86}Rb, was studied from solutions of rubidium chloride at a concentration of 0·5 or 5·0 meq./l. in the presence and absence of 10^{-4}M.2:4-dinitrophenol (DNP) at 0·2 and 25° C. To enable the quantity of rubidium which had been absorbed in a readily 'exchangeable' form to be estmated, the disks were separated from the solution at the end of the appropriate absorption period, blotted as uniformly as possible, and transferred to tubes containing inactive RbCl (10·0 meq./l.) at the same temperature. After exchange had taken place the disks were separated from the solution, which was assayed to determine the quantity of 'exchangeable' ions which the tissue had absorbed. The 'non-exchangeable' fraction was measured by assaying the disks. Results are expressed as microequivalents per gramme fresh weight.

Although it is convenient to describe the two fractions identified in these experiments as 'exchangeable' and 'non-exchangeable', no absolute meaning can be given to the terms. There is evidence that all monovalent ions within a tissue may be exchangeable if the times are sufficiently prolonged (MacRobbie and Dainty, 1958). Thus, if the experimental conditions were considerably changed, the relative magnitudes of the two components might well vary. The terms are here used solely to describe the situation observed in these experiments.

The duration of the absorption period and the period of exchange were varied between experiments. In all experiments batches of 20 disks weighing approximately 1·3 grammes were used in 50 ml. of solution, which was aerated throughout the experimental and exchange periods. The disks were equilibrated at the experimental temperature in distilled water for 30 minutes before active solutions were added; it had been found that the quantity of ions subsequently absorbed was unchanged when this period was varied from 30 to 120 minutes at both temperatures.

RESULTS

Since the object of these experiments was to ascertain the effects of temperature and of other conditions on the quantities of ions absorbed into 'exchangeable' and 'non-exchangeable' forms, preliminary experiments were carried out to ascertain appropriate experimental conditions.

Disks which had been allowed to absorb labelled Rb for 60 minutes at 0·2 or 25° C. were transferred to unlabelled solutions of RbCl containing 10·0

meq./l. at the same temperature. The quantity of ions which were exchangeable was determined after 15, 30, and 60 minutes (Table I). Since no significant change in the quantity of ions which exchanged occurred after 30 minutes, this period was therefore used for determining the 'exchangeable' fraction, the ions remaining in the tissue being described as 'non-exchangeable'.

TABLE I

Effect of the duration of exchange on the magnitude of the 'exchangeable' and 'non-exchangeable' fraction of rubidium in disks of carrot parenchyma after uptake for 60 minutes from a solution containing 0·5 meq./l. of rubidium choride

(*Results are expressed as μeq./g. fresh weight*)

Duration of exchange (minutes)	Temperature	Exchangeable fraction	Non-exchangeable fraction
15	25° C.	3·40±0·12	0·831±0·045
30	,,	3·64±0·11	0·670±0·063
60	,,	3·63±0·22	0·667±0·039
15	0·2° C.	3·23±0·07	0·477±0·064
30	,,	3·44±0·13	0·367±0·021
60	,,	3·37±0·13	0·353±0·131

In further preliminary experiments the appropriate duration of the period of absorption was considered. When disks were allowed to absorb Rb from solutions containing 0·5 meq./l. RbCl for periods of up to 6 hours it was found that their content of 'exchangeable' ions rose steeply over the first 30 minutes, when over 94 per cent. of the final value was attained; accordingly, an absorption period of 60 minutes was considered appropriate for the study of the 'exchangeable' fraction.

In subsequent experiments the following treatments were combined factorially:

Temperature: 0·2 and 25° C.
Dinitrophenol: 10^{-4} M. and nil.
Rubidium chloride: 0·5 and 5·0 meq./l.

The results of three identical experiments are given in Table II; an analysis of variance showed no significant differences between them. Significant effects and interactions are given in Table III, and the mean results are illustrated in Fig. 1. The 'exchangeable' and 'non-exchangeable' fractions were reduced by the lowering of temperature and by DNP; in the majority of cases these effects were statistically significant (Table III). However, the magnitude of the effects of temperature and of DNP on the two components contrasted markedly.

The analysis of variance shows (Table III) that at the lower concentration of rubidium there was no significant interaction between temperature and DNP, the effect of these two treatments being additive. At the higher concentration of rubidium, however, there were interactions between temperature

and DNP with respect both to the 'exchangeable' and to the 'non-exchangeable' fractions. It is apparent from Tables II and III that the 'exchangeable' fraction was reduced by the simultaneous application of DNP and the lowering of temperature to a greater extent than the sum of the individual effects of these

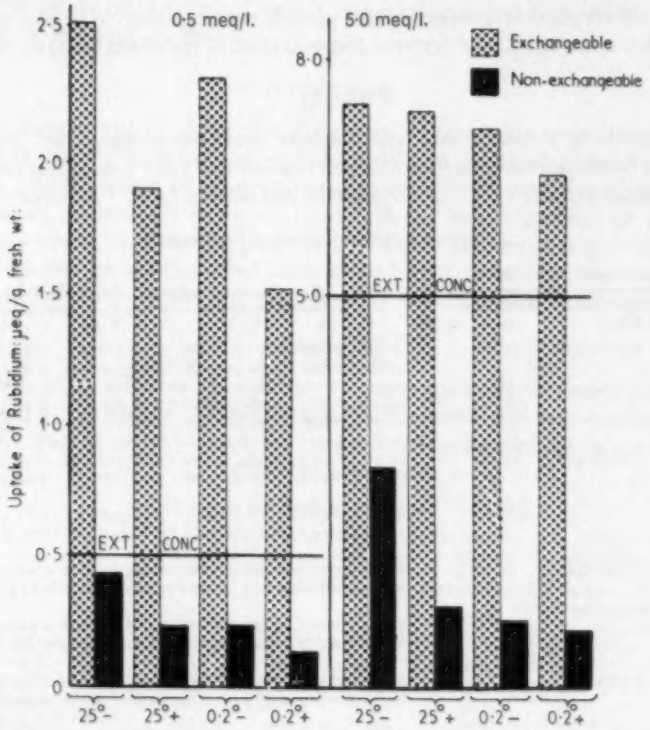

FIG. 1. The uptake of rubidium into 'exchangeable' and 'non-exchangeable' forms by disks of carrot parenchyma from solutions containing 0·5 and 5·0 meq./l. of rubidium chloride at 0·2 and 25° C in the presence (+) and absence (−) of 10^{-4} M. dinitrophenol. External concentration shown.
(Mean results of 3 experiments.)

two treatments when applied separately; conversely, low temperature or DNP alone reduced the 'non-exchangeable' fraction to almost the same extent as when the two treatments were applied together.

It is of interest to compare results for the extreme treatments, namely, 25° C. in the absence of DNP and 0·2° C. in its presence at the two concentrations of rubidium (Table III). Despite the fact that the quantity of 'exchangeable' ions absorbed from the higher concentration was nearly 3 times that absorbed from the lower, the combination of DNP and low temperature reduced this component to approximately the same extent, irrespective of the external

concentration of rubidium. In contrast, the quantity of ions held in the 'non-exchangeable' form was reduced to very different absolute amounts at the two concentrations of rubidium but the percentage reductions varied little.

Since it had been found that ions were held in 'non-exchangeable' forms at 0·2° C. to an appreciable extent, the time course of this process was investigated. Absorption was measured for periods ranging from 10 to 360 minutes at both 0·2 and 25° C., the external concentration of rubidium being 0·5 meq./l.

TABLE II

The uptake of rubidium into 'exchangeable' and 'non-exchangeable' forms by disks of carrot parenchyma from solutions containing 0·5 or 5·0 meq./l. of rubidium chloride at 0·2 and 25° C. in the presence and absence of 10^{-4} M. dinitrophenol

(Results are expressed as μeq.g. fresh weight)

	Experimental conditions			Experimental number			
	Concentration of RbCl	Temperature	DNP	C.T.10	C.T.11	C.T.12	Mean
Exchangeable ions	0·5 meq./l.	25° C.	−	2·35±0·028	2·41±0·080	2·81±0·053	2·53±0·053
			+	1·82±0·028	1·89±0·058	2·00±0·099	1·90±0·062
		0·2° C.	−	2·02±0·025	2·26±0·067	2·72±0·050	2·33±0·047
			+	1·37±0·008	1·49±0·023	1·67±0·033	1·51±0·021
	5·0 meq./l.	25° C.	−	7·03±0·017	7·23±0·100	7·88±0·102	7·38±0·073
			+	7·10±0·077	7·07±0·208	7·54±0·071	7·24±0·119
		0·2° C.	−	6·78±0·050	6·87±0·197	7·67±0·054	7·11±0·100
			+	6·24±0·095	6·55±0·126	6·81±0·088	6·53±0·103
Non-exchangeable ions	0·5 meq.l.	25° C.	−	0·399±0·008	0·380±0·009	0·495±0·012	0·424±0·010
			+	0·213±0·001	0·232±0·004	0·226±0·007	0·224±0·004
		0·2° C.	−	0·204±0·011	0·200±0·013	0·278±0·008	0·227±0·011
			+	0·112±0·002	0·125±0·002	0·141±0·004	0·126±0·003
	5·0 meq./l.	25° C.	−	2·550±0·044	2·830±0·010	2·783±0·032	2·721±0·029
			+	0·977±0·007	0·979±0·022	1·026±0·026	0·994±0·018
		0·2° C.	−	0·827±0·020	0·731±0·020	0·858±0·018	0·805±0·019
			+	0·625±0·025	0·649±0·012	0·681±0·023	0·651±0·020

Strikingly contrasting time courses in the 'non-exchangeable' fractions were observed at the two temperatures (Fig. 2). At 25° C. the 'non-exchangeable' fraction rose steeply and at a relatively constant rate throughout 360 minutes. At 0·2° C., however, ions entered 'non-exchangeable' forms considerably more rapidly over the first 60 minutes than subsequently, when a slow continuous uptake occurred. It appears, therefore, that at low temperatures an initial rapid binding in a 'non-exchangeable' form is followed by a slow continuing process. Regression equations for the linear parts of both curves, after 60 minutes, are:— 25° C : $y=0{\cdot}28+0{\cdot}006x$; 0·2° C : $y=0{\cdot}23+0{\cdot}001x$. When extrapolated to zero time the intercepts of the two regressions on the y axis are relatively close together. This suggests that at 25° C there may have been an initial rapid binding comparable to that at 0·2° C. From the regression

equations the following values for the Q_{10} can be calculated: at 60 minutes, 1·5; at 360 minutes, 2·3. These are compatible with diffusion being an important factor controlling the initial influx while the subsequent steady entry depends on concurrent respiration. More critical study of the effects of temperature on the binding of ions in non-exchangeable forms during short periods is, however, necessary.

DISCUSSION

The present results show that the extent to which the rubidium ion is bound in carrot tissues both in 'exchangeable' and 'non-exchangeable' forms can be reduced, either by the lowering of temperature or by the application of respiratory inhibitors. Consequently, it does not appear justified to assume that ions which have entered plant tissues in consequence of purely physical processes can be distinguished from those which have been metabolically accumulated solely on the grounds that they remain readily exchangeable.

TABLE III

Summary of the effects of the reduction of temperature and the presence of dinitrophenol on the uptake of rubidium into 'exchangeable' and 'non-exchangeable' forms by disks of carrot parenchyma from solutions containing 0·5 or 5·0 meq./l. of rubidium chloride

(*Mean results for experiments given in Table II.*)

	Reduction in exchangeable fraction		Reduction in non-exchangeable fraction		Statistical significance (P)	
	μeq.g.	per cent.	μeq.g.	per cent.	Exchangeable fraction	Non-exchangeable fraction
Effect of low temperature						
0·5 meq./l. −DNP	0·20	7·9	0·197	46·7	N.S.	<0·001
0·5 meq./l. +DNP	0·39	20·6	0·098	43·7	<0·01	<0·1
5·0 meq./l. −DNP	0·27	3·8	1·916	70·3	<0·05	<0·001
5·0 meq./l. +DNP	0·71	9·7	0·343	34·5	<0·001	<0·001
Effect of DNP						
0·5 meq./l. 25° C.	0·63	24·9	0·200	47·1	<0·001	<0·001
0·5 meq./l. 0·2° C.	0·82	35·2	0·101	44·4	<0·001	<0·05
5·0 meq./l. 25° C.	0·14	1·9	1·727	63·5	N.S.	<0·001
5·0 meq./l. 0·2° C.	0·58	8·1	0·154	19·2	<0·001	<0·01
Interaction of low temperature and DNP						
0·5 meq./l.	1·02	40·2	0·298	70·0	N.S.*	N.S.*
5·0 meq./l.	0·85	12·0	2·070	76·0	<0·05*	<0·001*

* Significance of interaction.

It is widely held that ions which enter into tissues without the expenditure of respiratory energy can be assigned to one or other of two categories, namely, those in the 'water free space' and those held in the 'Donnan free space' (Briggs *et al.*, 1958). The present results suggest that the negatively charged static sites which retain cations in the 'Donnan free space' are of two types, namely,

those which are labile and maintained only by current metabolism and those which are more permanent. Thus in carrot tissue, at any rate, the 'Donnan free space' is not confined to the cell walls and other inert material; on the contrary an appreciable component of the 'Donnan free space' must be due to negatively charged sites in the cytoplasm. Furthermore, the fraction of the

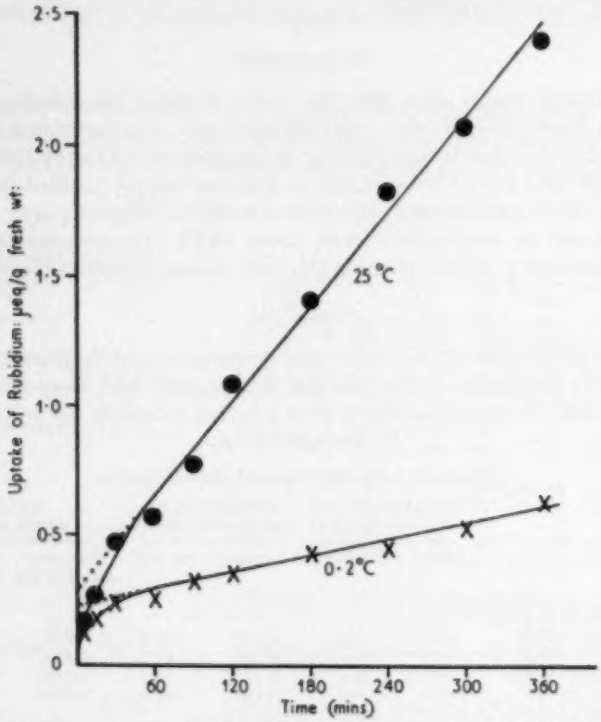

FIG. 2. The time course of uptake of rubidium into a 'non-exchangeable' form by disks of carrot parenchyma from a solution containing 0·5 meq./l. of rubidium chloride at 0·2° and 25° C.

'exchangeable' ions which are held by metabolically maintained sites in the cytoplasm appears to vary greatly depending on the external concentration; at an external concentration of 0·5 meq./l. it appears to account for some 40 per cent., but at 5·0 meq./l. only some 10 per cent. of the total 'exchangeable' ions are absorbed. Evidence that cells possess a considerable capacity to restrain ions in Donnan systems which are not dependent on concurrent metabolism is, however, provided by the fact that in the presence of inhibitors and at low temperature the concentration of 'exchangeable' ions in the tissues consistently exceeded that in the ambient medium.

It is of interest that in a recent study of ion transport in the alga *Nitellopsis obtusa*, MacRobbie and Dainty (1958) have postulated the existence of three

kinetic 'compartments' in which potassium may be retained, namely 'free space', 'protoplasmic non-free space', and cell sap. Ions in the 'free space' are assumed to exchange very rapidly; the half-time for exchange of ions in the 'non-free space' was considered something less than 30 minutes, while that regarded as in the cell sap exchanged very slowly. The compartment they describe as 'protoplasmic non-free space' would appear to be comparable to the 'exchangeable' fraction controlled by metabolism which has been identified in the present study, though no data on the effect of current metabolism on 'protoplasmic non-free space' was reported by them. More recently Diamond and Solomon (1959) have produced results using *Nitella axillaris* which are in many respects similar to those of MacRobbie and Dainty.

The effects of the reduction of temperature and the application of DNP on the 'non-exchangeable' component observed in the present investigation may now be considered. The marked reduction caused by both treatments is readily compatible with the view that 'non-exchangeable' ions have entered into an active transport mechanism. Whether the small quantities of ions which were bound in a 'non-exchangeable' form after the lowering of temperature or the application of the respiratory inhibitor can be attributed to active transport continuing, though at a greatly reduced rate, or whether ions can be bound in a 'non-exchangeable' form by other mechanisms is not clear. The data presented in Fig. 2 encourage the suggestion that at low temperature the binding of ions in a 'non-exchangeable' form can be divided into a fast and a slow component. This, however, does not establish the fact that two independent mechanisms are involved. That result, even if substantiated by more critical experiments, could be explained by the assumption that tissues which have been retained in distilled water for an appreciable period possess a certain concentration of a component which mediates active transport and that at low temperatures the replacement of this initial component is slow but continuing. Laties (1959) has recently drawn attention to the danger of assuming that metabolic processes are completely inhibited at low temperatures, and though the data which he cites are in the main concerned with temperatures appreciably higher than those used in the present investigation (5°C. as opposed to 0·2° C.), there appear no grounds for postulating that any mechanism, other than that responsible for active transport, rendered rubidium into a form which was 'non-exchangeable' under the present experimental conditions.

Finally, the possible relationship may be considered between the 'exchangeable' ions which are held in tissues in consequence of concurrent metabolism and those ions which have been rendered 'non-exchangeable' by active transport. On the one hand they could be regarded as two physiologically unrelated fractions; on the other it could be envisaged that ions are rendered *progressively* less exchangeable after their entry into the mechanism responsible for active transport. The view that the two components are controlled by independent metabolic mechanisms is at first sight encouraged by the fact that, whereas the combination of low temperature and inhibitors reduced the 'exchangeable'

fraction by a relatively constant amount, it reduced the 'non-exchangeable' ions by a relatively constant percentage (Table III). Alternatively, however, this situation could be due to ions in the initial stages of the active transport process being rapidly exchangeable, while those which have progressed more than a certain distance along this pathway are not. Although such a situation would be difficult to reconcile with the view that active transport depends on the function of specific carrier-ion complexes (Epstein, 1956), it is readily compatible with the suggestion that active transport may depend on the progressive transfer of ions between spatially orientated sites (Middleton and Russell, 1958). While there is no clear evidence in this regard, some features of the present results suggest that the distinction between 'exchangeable' and 'non-exchangeable' ions is much less fundamental than is commonly supposed. At the two concentrations of rubidium the combination of DNP and low temperature had comparable effects on both the 'exchangeable' and 'non-exchangeable' fractions. However, there was a positive interaction between DNP and temperature with respect to the 'non-exchangeable' fraction at the higher concentration of rubidium, though no interactions occurred at the lower. This situation encourages inquiry as to whether the ease with which ions exchange is determined not only by the nature of the sites in a tissue on which they are located, but also by the ambient ionic concentration. If this is so, ions which have entered active transport processes cannot be categorically identified solely on the basis of their exchangeability.

In common with many other investigations of salt uptake this study adds little positive information to our understanding of the mechanism of active transport. It appears, however, that some of the ions within a tissue which are readily exchangeable with those in the ambient medium are restrained in consequence of concurrent metabolism and that the readiness with which ions exchange does not necessarily provide a criterion for determining the type of mechanism whereby they have been absorbed.

LITERATURE CITED

AYLAND, M. J. (1956). *The uptake of nutrients by plants with special reference to monovalent ions.* B.Sc. Thesis, Oxford University.

BRIGGS, G. E., HOPE, A. B., AND PITMAN, M. G. (1958). Exchangeable ions in beet disks at low temperature. *J. exp. Bot.* **9**, 128.

DIAMOND, J. M., AND SOLOMON, A. K. (1959). Intracellular potassium compartment in *Nitella axillaris*. *J. Gen. Physiol.* **42**, 1105.

EPSTEIN, E. (1956). Mineral nutrition of plants: Mechanisms of uptake and transport. *Ann. Rev. Plant Physiol.* **7**, 1.

FRIED, M., AND NOGGLE, J. C. (1958). Multiple site uptake of individual cations by roots as affected by hydrogen ion. *Plant Physiol.* **33**, 139.

LATIES, G. G. (1959). Active transport of salt into plant tissue. *Ann. Rev. Plant Physiol.* **10**, 87.

MACROBBIE, E. A. C., AND DAINTY, J. (1958). Ion transport in *Nitellopsis obtusa*. *J. Gen. Physiol.* **42**, 335.

MIDDLETON, L. J., AND RUSSELL, R. S. (1958). The interaction of cations in absorption by plant tissues. *J. exp. Bot.* **9**, 115.

RUSSELL, R. S., AND AYLAND, M. J. (1955). Exchange reactions in the entry of cations into plant tissues. *Nature*, Lond., **175**, 204.

SUTCLIFFE, J. F. (1954). The exchangeability of potassium and bromide ions in cells of red beetroot tissue. *J. exp. Bot.* **5**, 133.

Cell Growth and the Structure and Mechanical Properties of the Wall in Internodal Cells of *Nitella opaca*

I. WALL STRUCTURE AND GROWTH

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WITH FOUR PLATES AND SIX FIGURES IN THE TEXT

Received 17 October 1960

SUMMARY

This is the first of two papers dealing with the relationship between growth and the mechanical properties of the wall in internodal cells of *Nitella opaca* L.

The submicroscopic structure of the cell wall of this alga, as determined by chemical analysis, X-ray crystallography, polarizing microscopy, electron microscopy, swelling measurements, and infra-red spectrography, is described in detail and the changes during growth are recorded. It has been found that the wall contains cellulose in the form of cellulose I (type B). The constituent microfibrils are preferentially oriented, usually in slow helices with considerable angular dispersion about the common direction. They are arranged in discrete layers with pectic substances providing an amorphous matrix between microfibrillar-reinforced laminations. It is shown that, as the cell elongates, both the streaming direction in the cell and the mean microfibrillar orientation in the wall change in such a way as to allow the possibility of a causal connexion between streaming and microfibrillar orientation in a new wall lamella. The orientation in such a lamella is undoubtedly modified by subsequent passive extension much as implied in the multi-net growth hypothesis of Roelofsen.

INTRODUCTION

THE elegant work of Green and Chapman (1955) and Green (1954, 1958) has shown that the internodal cells of *Nitella* are most convenient objects for the study of wall structure and growth. These cells, moreover, are so large that the physical properties of the walls can also be measured with comparative ease. There seemed therefore reason to believe that these plants would form ideal material for a critical study of the interrelationships between growth and the physical properties of the wall, and the present series of papers presents the results of an investigation of this kind made during 1958 and 1959. Whereas Green worked with *Nitella axillaris*, the only species available for the present work was *Nitella opaca*, and it was therefore thought desirable first to check the wall structure in our species against that used by Green. As already briefly reported (Probine and Preston, 1958), certain significant

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differences have been found between the two species; in addition, it has been found possible to make observations with *Nitella opaca* which Green found impossible with *Nitella axillaris*. It is therefore the purpose in this first paper to describe the wall structure of *Nitella opaca*. The mechanical properties of the walls of growing cells will be dealt with in the second paper, and it will then be shown that the significance of correlations between these properties and cell growth can be examined in more detail than has so far proved possible with any other cell. Although, therefore, features of structure will be described below which are clearly concerned with growth, discussion of their significance will be postponed to the second paper.

The internodal cells of *Nitella* have long been objects of interest in the field of plant physiology. It seems therefore rather odd that up to now it has not been known with certainty that the walls contain cellulose. Although both Correns (1893) and Votava (1914) claimed that the *Nitella* cell wall contains a substance giving the staining reactions of cellulose, Debski (1898) was not able to identify the skeletal substances. Even Green (loc. cit.) does not offer any evidence of his own on the composition of the wall and, on the basis of the early work referred to above, merely assumes that the birefringence of the wall is due to a cellulose. Again, Nicolai and Preston (1952) included *Nitella* in a survey of the fine structure of the walls in about sixty species of filamentous green algae, using chiefly the methods of X-ray diffraction analysis, but found that the diagram was much too poor in diffraction arcs to allow a decision to be made on the nature of the skeletal substance. This is understandable in the light of the present investigation since it has been shown that some pretreatment of the wall is necessary before a satisfactory X-ray diagram is obtained.

The only information on the chemical nature of the wall, therefore, has rested on the evidence of staining tests. In view of the unreliability of staining and solubility tests for native cellulose (Nicolai and Preston, 1952) it was considered desirable to begin this investigation by an examination of the nature of the structural polysaccharide by the methods now available.

MATERIALS AND METHODS

The morphology of *Nitella* has already been reviewed by Green and Chapman (1955) but those features relevant to the present study will be described here for the sake of clarity.

The plant consists of a linear series of successive nodes and internodes. The nodes are multi-cellular with lateral cells of limited and of unlimited growth. The internodes, with which this paper is solely concerned, are single cylindrical cells which after being cut off from the apex proceed to elongate, without proportional increase in diameter, up to a length of several centimetres. Green (1954) has shown conclusively that growth in these cells is evenly distributed over the whole cell length. The walls of these elongated internodal cells are lined with cytoplasm surrounding a large central vacuole.

Within this parietal cytoplasm and close against the wall lies a single layer of densely packed discoid chloroplasts arranged in files slightly tilted from the cell axis in a steep helix. These chloroplasts are, however, lacking along two files on opposite sides of the cell which also pass along the cell in the same steep helix. In living cells the cytoplasm may be seen to be streaming along the direction of the files of chloroplasts, ascending on one side of the clear region and descending on the other, so that the direction of streaming may easily be measured as the direction of the files of chloroplasts. These clear regions, which are referred to as *striations*, thus represent the boundaries between the ascending and the descending streams.

In the present paper the wall is examined chemically by hydrolysis followed by paper partition chromatography and physically by the methods of polarization microscopy, X-ray diffraction analysis, infra-red absorption spectrophotometry, and electron microscopy. For chemical analysis of the wall, whole plants were treated according to the methods of Cronshaw, Myers, and Preston (1958), modified from Jermyn and Isherwood (1956). Otherwise cell walls were isolated by cutting away each end of the internodal cell and brushing out the cytoplasm from the hollow cylinder thus produced. For some observations it was found necessary to subject the walls to chemical treatments and these will be described in the appropriate place. Determinations of major extinction positions were made by the standard method using a Red I plate. For X-ray analysis, CuK_{α} radiation was used, collimated to a beam 0·5 mm. diameter with a specimen/film distance of about 3 cm., calibrated accurately against a silver diagram. For electron microscopic observation the wall was either ground in a blender or dissected into lamellae as described in the body of the paper. A few observations were made on ultra-thin sections imbedded in the usual way in a mixture of methyl and butyl methacrylate.

RESULTS

(*a*) *The Identification of the Skeletal Material as Cellulose*

The first step in the determination of structure was the chemical fractionation of the wall and the identification of the sugars produced by hydrolysis of the various fractions. Identification was achieved by comparison with the positions and colours of standard sugars run on the same chromatogram, and the results are given in Tables I and II.

TABLE I

Proportion of various constituents in the wall

(*per cent. dry weight*)

Water-soluble fraction	29·9
Alkali-soluble fraction	34·8
Chlorite-soluble fraction	18·6
α-cellulose	16·7

TABLE II

Sugars in the hydrolysis products of wall fractions

	Water-soluble fraction	Alkali extract	α-cellulose
Galacturonic acid	M	—	trace
Galactose	M	V.W.	—
Glucose	S	S	S
Mannose	V.W.	V.W.	W
Xylose	trace	M	W
Rhamnose	trace	trace	—

S (strong); M (moderate); W (weak); V.W. (very weak).

The water-soluble fraction of the wall amounts to about a third of the initial dry weight. Although this is a fairly large fraction of the total wall material, it is, nevertheless, small compared with that of most of the algae examined by Cronshaw, Myers, and Preston (1958). Comparable figures for the water-soluble fraction in that investigation were of the order of 50 to 70 per cent. Only *Cladophora*, *Enteromorpha*, and *Ptilota* had water-soluble fraction below 40 per cent. Galacturonic acid in the water extract indicates the presence of pectic substances in the wall. There is X-ray evidence that the strong glucose spot is due partly to hydrolysis of starch (p. 265). The α-cellulose fraction (17 per cent.) is not as high as that of *Cladophora* (28·2 per cent.), *Chaetomorpha* (41 per cent.), and, particularly, *Valonia* (75 per cent.). It is, however, comparable with *Enteromorpha*, *Ulva*, *Laminaria*, *Ptilota*, and *Griffithsia*. The fact that sugars other than glucose occur in the hydrolysate of this fraction (which was observed in the electron microscope to contain microfibrils only), puts *Nitella* outside the unique group which yield only glucose (Myers and Preston, 1959), though the trace of galacturonic acid has possibly been carried over from earlier stages in the extraction.

At each stage of the chemical treatment a small sample of the residue was examined by X-ray diffraction analysis. The principal spacings determined from the X-ray diagrams of powdered wall material are listed in Table III in order of increasing intensity. The table also includes corresponding accepted spacings of cellulose I and II and of starch. The diagrams will be discussed in reverse order. The X-ray spacings of the final residue ('post chlorite', col. 7) are similar to those of cellulose II, both in value and in the relative intensity of the corresponding arcs. This close agreement, taken with the fact that glucose is the principal sugar in this material, undoubtedly means that this residual component must at least be something like cellulose II. Although the spacing at 9·6 Å. is longer than any accepted for cellulose, spacings of this order have been recorded in the cellulose both of higher plants (Sen and Woods, 1948; Preston and Singh, 1950) and of algae (Myers, Preston, and Ripley, 1956; Cronshaw, Myers, and Preston, 1958).

The diffraction diagram of the material after alkali treatment (Table I, col. 6) shows much more background scatter than was present on the 'post chlorite' diagram, indicating the removal of much amorphous material by

chlorination. There is, however, little doubt that at this stage the crystalline material is already cellulose in the form of cellulose II.

After extraction of the water-soluble material (Table III, col. 3), the spacings compare very favourably with the principal spacings of cellulose I (with the exception of the long spacing at 8·9 Å., to which the remarks on long spacings above also apply). There is no evidence of the presence of cellulose II; for instance, the ring at 7·35 Å. is absent and it will be shown below that the spacing listed at 5·25 Å. refers to 10$\bar{1}$ planes, consistent with the 5·39 (10$\bar{1}$) of cellulose I but not the 5·19 (020) of cellulose II. The indications are, clearly, that the crystalline substance present after removal of the water-soluble material and before the alkali treatment, is something like cellulose I. Treatment with 4N. KOH converts this to something very like cellulose II.

TABLE III

Principal spacings in order of intensity calculated from powder diagrams

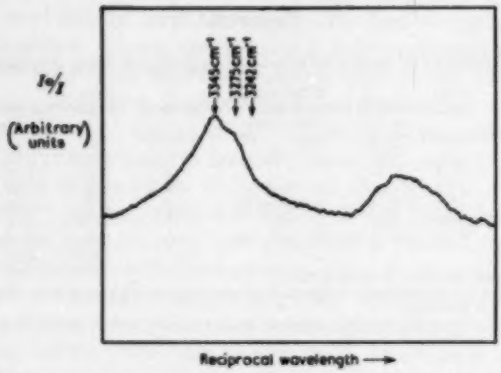
Post-alcohol	Starch-B	Post-water	Cellulose I	Miller index cellulose I	Post-alkali	Post-chlorite	Cellulose II	Miller index cellulose II
5·18	5·17 *s*	3·90	3·93	002; 301	4·41•–4·46•	4·39	4·42; 4·38	002; 021
16·00	15·6 *s*	4·25	4·34	021	4·03	4·01	4·03	002
4·00	4·00 *m*	8·94	—	—	7·35	7·23	7·35	101
8·33	—	5·25	5·39	10$\bar{1}$	—	3·10	3·14	031; 131; 130
6·11	6·29 *m*; 5·89 *m*	2·58	2·58; 2·59	040; 212; 311	2·58	2·58	2·58	040
4·43	4·51 *m*	2·14	5·97	101	2·13	2·13	2·21	202; 103
2·58	—	6·10	1·95; 2·16	004; 240; 042	—	9·64	—	—
3·69	3·68 *m*				—	5·15	5·19	020

Notes: (1) The spacings marked • are very diffuse.
(2) The 4·42 and 4·38 spacings of cellulose II are not resolved and have been bracketed.
(3) *s*, strong; *m*, moderate.
(4) The order of intensity is approximate only and is intended to serve as a guide to identification, and not as a criterion.

The X-ray powder diagram of the material after the alcohol extraction (col. 1) resembles closely that of starch type B (col. 2) (Bear and French, 1941), the type of starch known to be present in some algae (Frei and Preston, 1960). It seems likely, therefore, that the cellulose I diagram has here been partially masked by a starch diagram type B. The absence of starch diagram after boiling in water and the presence of glucose in the hydrolysate of the water extract can be regarded as confirmatory.

The general conclusion reached from inspection of Table III is, therefore, that the crystalline component of the wall is cellulose I. The comparison between the *Nitella* skeletal material and *Cladophora* eucellulose illustrated in the sector diagram in Pl. I, Fig. 1, fully confirms this view. It is clear that there is complete correspondence of spacings within the limits of resolution of the photograph. There appear, however, to be some slight differences between the two diagrams, in that the relative intensities of reflection from the various crystal planes are different. In particular, the difference between the intensity of the reflection from the 101 planes (inner ring), and the intensity of that from the 002 planes (fourth ring from centre) appears to be greater in *Cladophora* than it does in *Nitella*. Similarly, reflection from the 230 plane is stronger, relative to the intensity of that from the 040 plane, in *Cladophora*

than in *Nitella*; but the combined reflection from the 130, 031, and 131 planes is stronger relative to that from the combined 221 and 122 planes in *Nitella*. The reason for the difference in relative intensity of the rings is not clear. It may be due to a difference in molecular packing in the crystal or to a difference in degree of crystal perfection which might be associated with the presence in *Nitella* cellulose of mannose and xylose. Whatever the cause it is probably not due to a major difference in crystal structure. One other point should be mentioned. The rings are much more diffuse in the *Nitella* diagram than in the *Cladophora* diagram. This, again, might be due to a less perfect crystal structure in *Nitella* or to the presence of much smaller crystallites. The very great deal of amorphous scattering indicates that the wall is not highly crystalline.



TEXT-FIG. 1. Trace of infra-red absorption spectrum of a wall sample prepared, as in text, after deuteration. This shows the presence of cellulose I and the complete absence of cellulose II

In view of these (minor) differences in the detail of the X-ray diagrams of eucellulose and *Nitella* cellulose, it was thought desirable to confirm the presence of cellulose I by infra-red absorption spectrophotometry. Fresh cells, from which the protoplasm had been removed, were reduced to a fine suspension in water in a blender using a Teflon pestle, and this was smeared on to a microscope slide and allowed to dry. The quantities of water and wall material were so adjusted that the mean density of the dry film was 3 μg./mm.[2] After the film had been dried it was removed from the slide with a sharp razor blade and examined as described by Marrinan and Mann (1954, 1956) and Mann and Marrinan (1956).[1] The results are shown in Text-fig. 1. After a vapour deuteration the material gives a cellulose I, Type B spectrum (as given by cotton, linen, &c.). The prominent band at 3,242 cm.$^{-1}$, typical of a Type A spectrum (typical of the eucellulose of *Valonia*) is absent and there is

[1] The infra-red measurements were made by Dr. J. Mann in the laboratories of The British Rayon Research Association (Manchester).

no evidence of any cellulose II. The amount of material deuterated indicates a high proportion of amorphous material, and this too is consistent with qualitative X-ray evidence.

(*b*) *Orientation of the Cellulose*

(1) *X-ray diffraction analysis*

Determination of the orientation of the crystallites in the wall by X-ray analysis should preferably be made on a single piece of wall oriented in the spectrometer with respect to some morphological axis of the cell. This would require impracticably long exposure times with *Nitella* since the wall is not highly crystalline. In this case, therefore, an accurately aligned and stacked bundle of single cell walls has been used, cut from the same cell. The beam was directed normal to the plane of the wall, with the longitudinal axis of the cell vertical (parallel to the length of the page in the figure). The material was pretreated with dilute acid to remove amorphous material and a front beam-stop[1] was used to reduce background scatter. The resulting diagram (Pl. I, Fig. 2) shows that there is some preferential orientation of the crystalline component. The (002), (10$\bar{1}$), and (101) arcs are strongest along the meridian so that the length of the crystallites must be preferentially oriented in an almost transverse direction. There is, however, a considerable angular dispersion about this mean direction.

Diagrams have also been obtained with the beam parallel to the face of the wall, (*a*) at right angles to the longitudinal axis (Pl. I, Fig. 3) and (*b*) parallel to the longitudinal axis. With the X-ray beam at right angles to the longitudinal axis, the 101 planes are represented by an equatorial arc and 10$\bar{1}$ planes are represented by a very faint meridional arc. This indicates that the 101 planes tend to be oriented parallel to the plane of the wall. The 10$\bar{1}$ reflection occurs only by virtue of the wide angular distribution of crystallites in the plane of the wall. With the beam parallel to the longitudinal axis, the position of the arcs is the same but the distribution of density around the arcs is quite different.

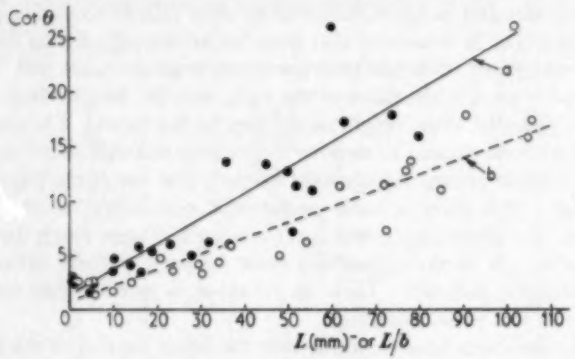
(2) *Light microscopy*

According to Green and Chapman (1955), the direction of the m.e.p.[2] in single walls of *Nitella axillaris* lies transversely to the cell axis so that the birefringence is negative. This is consistent with the above X-ray evidence. In *Nitella opaca*, however, the m.e.p. is not exactly transverse; it usually lies around the cell in a slow helix. This has already been fully documented elsewhere (Probine and Preston, 1958) and it has been accepted both by us and

[1] This is a small lead cup inserted between the specimen and film, intercepting the primary beam and therefore reducing air scatter.

[2] The 'm.e.p.' is the major extinction position of the wall when viewed between crossed nicols. It corresponds to the direction of the major refractive index and therefore to the mean direction of the cellulose crystallites.

by Green (1959) that there is in this respect a real difference between *Nitella axillaris* and *Nitella opaca*. Although the difference may be one of degree rather than of kind, it is not proposed at the moment to take this point further. As already reported by Probine and Preston (1958) there is, however, in *Nitella opaca* a change in the inclination of the m.e.p. as an internodal cell grows longer and therefore older, and this, as well as other changes also associated with length increase, will be briefly restated here. Perhaps the most noticeable, and certainly the simplest association is that between cell length and the angle (θ) which the streaming direction makes with the cell axis. It is



TEXT-FIG. 2. The relationship between cot θ (θ = angle between cell axis and streaming direction) and cell length (L) or ratio of length to breadth (L/b). Curve *a* (solid circles), L.; curve *b* (open circles) L/b.

convenient to plot the length against cot θ as in Text-fig. 2*a*. The relationship tends to be linear, with a scatter of the experimental results which is considerably reduced when the length/breadth ratio is used as abcissa in place of the length alone (Text-fig. 2*b*). It therefore follows that the streaming direction depends both upon the length and the breadth of the cell. The correlation coefficient between L/b and cot θ (0·91) is highly significant (0·62 at the 0·0001 level of significance for 23 degrees of freedom) and the regression equation is of the form

$$\frac{L}{b} = a + c \cot \theta,$$

where L and b are the length and diameter of a cell respectively and a and c are constants. A relationship of a more complex type occurs between the streaming angle (θ) and the direction (ψ) of the m.e.p. of the wall. The relationship has already been expressed graphically elsewhere (Probine and Preston, 1958) but it is more instructive to examine the numerical results themselves. These are presented in Table IV for cells of various known lengths and, therefore, ages. Although there is a highly significant correlation

TABLE IV

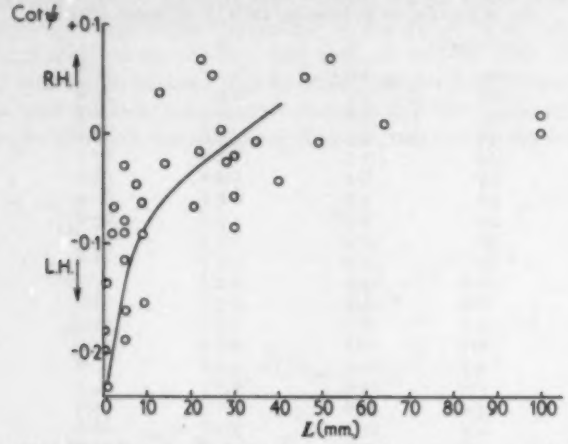
The angle between streaming direction and cell axis (θ), between the m.e.p. and the transverse plane (ψ), and the angular separation of the streaming direction and the m.e.p. (α) in internodal cells of different lengths

Cell length	θ	ψ	α
100	2·1 *r*	1·1 *r*	86·8
100	3·2	0·0	86·8
64	3·5	0·2 *r*	86·3
48	4·7	0·5 *l*	85·8
30	7·3	5·0 *l*	87·7
25	8·4	3·0 *r*	85·0
22	9·5	0·9 *l*	81·4
21	9·9	3·7 *l*	83·8
28	11·2	1·1 *l*	79·9
14	13·0	1·6 *l*	78·6
12·0	14·5	2·0 *l*	77·5
9·0	16·5	5·3 *l*	78·8
9·0	16·5	8·9 *l*	82·4
7·5	18·1	2·7 *l*	74·6
5·5	21·5	9·3 *l*	77·8
5·0	22·0	6·7 *l*	74·7
3·0	26·8	6·1 *l*	69·3
2·5	27·2	3·9 *l*	66·7
4·8	29·9	10·7 *l*	70·8
1·0	30·8	13·4 *l*	72·6
0·5	35·1	11·2 *l*	66·1
0·5	35·6	10·2 *l*	64·6

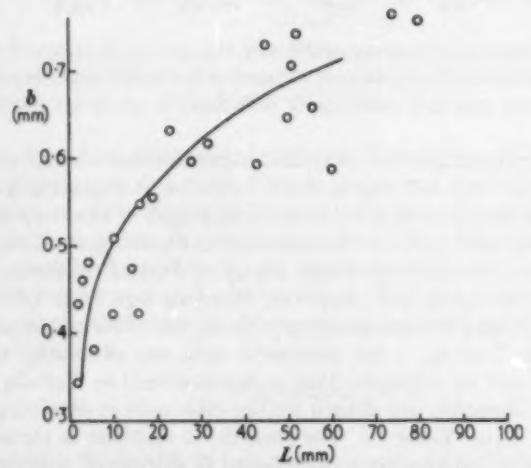
($r = 0{\cdot}85$) between streaming angle and the m.e.p. ($r = 0{\cdot}617$ at the 0·001 level of significance for 23 degrees of freedom), the fact that the angle between them (α) is not constant must imply that there is no direct geometrical connexion.

The close correlations thus described imply that there must be a correlation between m.e.p. and cell length. Such a relation is expressed graphically in Fig. 3 and is clearly complex. Up to a cell length of about 15 mm. ψ varies almost linearly with l, but in the sense that as the cell grows longer the spiral becomes flatter. Beyond this length the upward trend continues, though at a slower rate, the spiral now, however, changing sign from left-hand (S) to right-hand (Z) and becoming steeper. During the whole of the growth period illustrated in Text-fig. 3 the internodal cells are, of course, increasing in diameter as well as in length. This is demonstrated in Text-fig. 4 in which lengths and diameters are plotted for the cells used in obtaining the data in Figs. 2 and 3 and Table IV. The increase in diameter is throughout small compared with the increase in length and if changes in wall structure were determined by absolute changes in dimensions then it would be expected that length changes would be of overriding importance. This is clearly not the case. It may be significant, however, that the rate of change of diameter with length (db/dL, Text-fig. 5) is high for shorter cells and falls rapidly as the cells grow longer and therefore older. The helix defined by the m.e.p. thus becomes flatter at a time when db/dL is high and steeper when db/dL is low.

The possibility of a connexion between protoplasmic streaming and wall structure is strengthened by observation of the wall lying over the striation visible in the cytoplasm and referred to above. As already remarked upon by



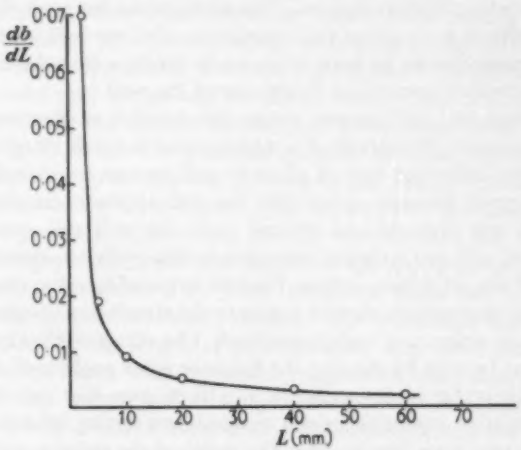
TEXT-FIG. 3. The relationship between cot ψ (ψ = angle between m.e.p. and the transverse plane) and L. R.H. = right-hand, L.H. = left-hand helix. The curve is drawn in arbitrarily.



TEXT-FIG. 4. The relationship between cell diameter b and cell length, L, in growing cells. Each point refers to a *different* cell.

Green and Chapman (1955) the position of the striation—associated with a discontinuity in flow—is marked by a linear region of the wall with optical properties different from those of the rest of the wall. Each striation is visible as a linear region which appears to extinguish, in the polarizing microscope,

when its long axis, rather than that of the cell, is parallel to the vibration plane of either polaroid. Movement of a striation through the extinction position reveals changes in brightness which are somewhat difficult to interpret. Many striations show a very narrow isotropic 'line' down the centre. While their properties are difficult to determine, there is no question that the striations differ structurally from the rest of the wall. The results of the



TEXT-FIG. 5. The relationship between the relative rate of breadth increase (db/dL) and cell length.

present investigation are so far in general agreement with those of Green and Chapman (1955), but there are some further observations which are relevant. In *Nitella opaca* the structural difference which occurs in the region of the striation is certainly a difference in microfibril arrangement, i.e. it is a discontinuity of arrangement of the crystalline component and not of the amorphous material. This is clear from the fact that, after a wall has been chemically treated,[1] lamellae stripped from it in the region of the striations and observed in the electron microscope show microfibrils with little or no amorphous material encrusting them (see below). When cells which have been treated in this way are examined in the polarizing microscope, however, the optically different striations are still clearly visible. Not all striations, however, are structurally similar. Some show the very narrow isotropic line down the centre mentioned by Green and Chapman (1955) but the common appearance between crossed nicols is as shown in Pl. I, Fig. 4. In these cases the striation shows up as a central bright 'line' bounded on each side by a dark 'line' when viewed with the microscope stage considerably displaced

[1] The cells were treated with hot 2 per cent. HCl for 30 minutes, washed, treated with hot 4 per cent. KOH, and washed. This cycle was repeated three times. They were then given a chlorite treatment as described by Cronshaw, Myers, and Preston (1958).

from the wall extinction positions. The presence of these dark lines, which do not extinguish sharply, suggests that, in these localized areas, the structure differs from the rest of the wall in one or more of the following ways:

(*a*) The wall might be much thinner, or be less crystalline. The fact that the wall separates into lamellae which are continuous sheets, and which do not appear to fail preferentially in these regions, does not reinforce this view.

(*b*) The microfibrils might be considerably disoriented about their mean preferred direction in these regions. This seems to be the more likely explanation. It is difficult to measure the retardation of these dark areas accurately, but such measurements as have been made indicate that the retardation is indeed considerably lower than in the rest of the wall.

The variation in birefringence across the striation is illustrated in Pl. II, Fig. 5, a composite photograph of a striation and the wall on either side of it, built up in the following way. A piece of wall was set to extinction, and the stage then turned through 45° so that the wall appeared bright. An elliptic compensator was inserted and rotated until the wall was extinguished. A photograph of wall and striation was taken in this position, and at 2° steps on either side of the extinction setting. Narrow strips of the same portion of wall cut from each photograph, at right angles to the striation, were then assembled side by side in order and rephotographed. The result is Pl. II, Fig. 5. The effect can best be seen by viewing the figure at a low angle to the plane of the page in the direction of the striation. It will be seen that the wall on either side of the striation extinguishes at a compensator setting (shown on the side) which is different from that at either the centre of the striation or in the bands on either side. These latter appear to have a lower birefringence than the rest of the wall, and the centre a slightly higher birefringence. This piece of wall was chosen because the striation and wall extinguished together between crossed nicols and the birefringence was of the same sign so that this is not likely to be an artifact due to these regions having different m.e.p.'s.

The nature of the structural discontinuity which occurs in the region of the striation appears to be somewhat variable and, therefore, difficult to define with precision. This topic will be discussed further after the electron microscopic evidence has been presented. At this stage it is merely noted that a definite structural discontinuity occurs at the boundary of the two cytoplasmic streams, consistent with the possibility that direction of streaming and microfibril direction might be linked. One further point might be made, however, before leaving this topic. It has been tacitly assumed in the foregoing discussion that the flow boundaries are more or less fixed in relation to the cell surface. There is good evidence that they do indeed occupy the same region of the cell surface throughout most of the life of the cell (Probine, 1959).

(3) *Wall swelling in water*

Anisotropy of swelling was used early as an indication of anisotropy of structure (Schwendener, 1874); and, more recently, attempts have been made to relate anisotropy of swelling to orientation in man-made fibres, see, e.g.

Hermans, 1949. In the present investigation measurements of the degree of swelling in the radial, transverse, and longitudinal directions in the cell wall of *Nitella* have yielded valuable information on the structure of the wall.

The increase in thickness of the wall (radial direction) due to wetting has been measured in two ways. In the first method a Philips displacement transducer (GM 5537) was used as a thickness gauge. In this instrument, linear displacement of a lightly loaded measuring stylus is converted into an electrical signal, and the magnitude of the displacement is indicated directly on the output meter of a direct-reading measuring bridge (GM 5536). For this application the transducer was held rigidly above the surface of a good precision-ground and lapped surface plate. A cell, from which the contents had been removed, was opened out flat and dried down on to a glass plate which was rigidly fixed to a precision-ground vee block movable over the surface of the plate in such a way that the stylus rested either on the glass reference surface, or on the specimen. The difference in the readings obtained in these two positions represented the dry thickness of the specimen. In practice, a reading was taken in the middle of the specimen and on the glass reference surface on either side of the specimen, these two latter readings being averaged to give the 'zero' reading. The procedure was repeated at several points along the length of the specimen. A drop of water was then placed on the specimen with the stylus in position and, after swelling had ceased, the bridge output meter indicated the increase in thickness directly. The results obtained from a number of specimens indicated that the increase in wall thickness on wetting was of the order of 100 per cent. The dry and wet thickness of a strip of wall was also measured directly by an optical method. This measurement was much more difficult and inconvenient and served only as a check on the transducer measurements. The increase in thickness was again of the order of 100 per cent.

In the transverse direction in the plane of the wall the increase in dimension on wetting, measured microscopically, was of the order of 4 per cent., and, in the longitudinal direction, 6 per cent.

The large percentage increase in the thickness of the wall on wetting suggests that there is little reinforcement of the wall in the radial direction, such as would occur, for instance, if the microfibrils were interwoven and entangled throughout the thickness of the wall. The observed behaviour of the wall is consistent with the idea that the microfibrils are arranged in discrete layers with pectic substances providing an amorphous matrix between microfibrillar reinforced laminations, or, at very least, that the microfibrils lie strictly in the plane of the wall. That the first of these models is substantially correct is shown by the fact that, when the wall is treated with hot 0·5 per cent. ammonium oxalate for 30 minutes to remove pectic substances, it can be separated into thin lamellae using a needle and fine forceps. In Pl. II, Fig. 6 a photograph is reproduced of a piece of wall which had been torn across and had failed in such a way that five separate wall layers are visible. The photograph was taken between crossed nicols so that differences in optical thickness show up clearly

and reveal the boundaries of the laminations. This is a new observation for *Nitella*. Green and Chapman (1955) recorded consistent failure of attempts to flake or tear the wall of *Nitella axillaris* into thin lamellae and concluded that the wall was uniform in composition. Apart from its structural importance and its bearing on the elastic symmetry of the wall, the fact that the wall can be separated into thin lamellae opens up new possibilities for more precise structural examination in the electron microscope.

(4) *Electron-microscopic examination*

Further chemical treatment is necessary, however, before the lamellae are sufficiently free of amorphous material for detailed study. The following treatment, which is a slight modification of one used by Kreger (1957), has been found satisfactory. The wall was treated successively with hot 2 per cent. HCl and hot 4 per cent. KOH and washed. This cycle was repeated three times. The wall was then given a chlorite treatment as described by Cronshaw, Myers, and Preston (1958). It could then easily be stripped under water into lamellae free of amorphous material when examined in the electron microscope.

An electron micrograph of a piece of wall so treated is reproduced in Pl. II, Fig. 7. In spite of the high magnification, the microfibrils show up quite clearly and cleanly. The microfibrils are unusually narrow, the finest being of the order of 50 Å. wide, compared with the 200 Å. or so thick microfibrils of *Valonia* (Preston *et al.*, 1949; Preston, 1951; Preston and Cronshaw, 1958). Among the other algae for which figures have been published, *Cladophora rupestris* has microfibrils 300–250 Å. wide and the alga with the narrowest microfibrils in the group listed by Cronshaw, Myers, and Preston (1958), was *Ulva lactuca* (180–90 Å.). Microfibrils 50–100 Å. wide have been recorded for conifer tracheids by Hodge and Wardrop (1950), and Rånby (1952) lists widths of 73–80 Å., 87–90 Å., and 108–160 Å. for wood cellulose, cotton cellulose, and tunicin respectively. *Nitella* microfibrils appear, therefore, to be on the lower limit of microfibril size. The small size of the crystallites was one of the reasons advanced above for the diffuse nature of the X-ray diagram of *Nitella*, and this electron-microscope evidence is qualitative confirmation of this suggestion.

Examination of stripped material also enables some estimate of the microfibril orientation to be made. The mean direction of the microfibrils with respect to the longitudinal axis cannot be determined with any very great accuracy by this means (within $\pm 20°$, say), as it is not always possible to orient accurately wall strips on the specimen grid. Reasonably reliable estimates can be made, however, of the distribution of microfibrils about the mean fibril direction—subject to the proviso that the act of stripping has not caused misalignment at the interface as the strip was pulled away.

An example of the appearance of an inner layer stripped from a young cell (length about 10 mm.) is shown in Pl. III, Fig. 8. The *approximate* direction

of the longitudinal axis is shown. It is apparent that the bulk of the microfibrils lie approximately at right angles to the longitudinal axis, in agreement with the X-ray and polarizing microscope evidence. There is, however, a fairly large number of microfibrils lying roughly at right angles to the main direction. It is almost as if this is a very imperfect example of a crossed-fibrillar structure. The possibility that the 'longitudinal' microfibrils are artifacts due to fibril displacement in the stripping process is unlikely for two reasons. First, there appear to be fibrils with longitudinal orientation lying deep within the wall with transverse fibrils overlying them. Secondly, Green (1958) has published replicas of the wall which show microfibrils considerably displaced from the transverse direction—particularly in his Pl. 247, Fig. 6. His replica technique was not likely to cause displacement of the microfibrils from the transverse direction, so that it must be concluded that, in some circumstances, the microfibrils can assume this peculiar orientation. Green notes that this particular photograph is of an internode approaching the end of its elongation. Pl. IV, Fig. 10, which is from an unknown location within the wall, also shows evidence of a crossed fibrillar pattern, but with considerable disorientation about both directions.

The angular frequency distribution of the microfibrils has been obtained in the following way. The central area of the micrograph shown in Pl. III, Fig. 8, was enlarged twofold and the enlarged print was repeatedly and randomly pierced with a pin all over the surface. The direction of the microfibril nearest each pin-point was determined and a histogram constructed showing the number of microfibrils having directions within the angular ranges, 0–10°, 10–20°, 20–30°, &c. relative to some fixed direction. The angular frequency distribution obtained in this way is shown on the diagram overlying the photograph. It is evident that there are two main directions. The method clearly tends to cause a bias in favour of the 'longitudinal' fibrils which appear, in general, to be overlying the 'transverse' fibrils. It will be recalled that the X-ray diagrams do not show the characteristic 'crossed-fibrillar' pattern which could therefore be expected. It seems likely, however, that the 'longitudinal' microfibrils occur very much less frequently than do the transverse. Further work remains to be done before this feature of the wall is completely understood. At this stage, attention is merely drawn to the apparent presence of a crossed-fibrillar structure.

In some cases the wall shows a series of disturbances (Pl. III, Fig. 9) such as have been described by Green (1958).

In Pl. IV, Fig. 11, an electron micrograph is reproduced of the extreme outside layer of an immature cell. This layer was notable in that, after the wall had been prepared for stripping, there was very little evidence of bonding between it and the rest of the wall and it was separated with very little manipulation indeed. In this case the microfibrils are aligned longitudinally and this was the case for all grids examined on which this particular layer was mounted. This agrees very well with what one would expect on the basis of the multinet theory of Roelofsen (1951) and Roelofsen and Houwink (1953).

Other outer layers showed this tendency to longitudinal orientation but this particular case provided the most notable example.

For electron microscopical observation of the wall in the region of the striation fairly mature cells (i.e. length 30 mm.) were chosen because optical studies suggested that the striation might be more easily recognized in these cells. The protoplasm was removed as previously described and each cell was cut into 2-mm. lengths. These were chemically treated for stripping and each cylinder was then opened out to form a flat sheet. A thin layer of wall was removed from the inner surface of the cell, and placed on a Philips grid so that the direction of the cell length was at right angles ($\pm 15°$) to the grid slot.

It was found that a striation always showed up as two electron-dense bands close together and easily recognizable (Pl. IV, Fig. 12). The identification of these bands with the striation region was based on the grounds that (*a*) the bands were always at right angles to the grid slot within the limits given, (*b*) where two pairs of such bands were visible they were parallel to each other and separated by a distance of the same order as half of the cell circumference, (*c*) on the one occasion when grid slot and longitudinal cell axis were parallel, a pair of electron-dense bands, present by chance, ran the whole length of the specimen, (*d*) the bands did not have the appearance of folds, which are easily distinguished, and (*e*) the separation of the electron-*dense* bands agreed very well with the separation of the *low* birefringence areas visible in the polarizing microscope.

In the bands there sometimes appear to be a number of microfibrils running parallel to the striation, but apart from this there seem to be no particular associated structures. On either side of the 'striation' area the wall appears quite uniform over some considerable distance from it and within this the microfibrils seem to lie roughly at right angles to the striation. The 'striation' areas of a number of cells were examined and in each case the electron-dense regions were easily distinguishable.

There is therefore very strong evidence that a discontinuity of wall structure does exist in the region of the striation. It would appear that the 'electron-dense' areas are regions of greater disorder than found either in the rest of the wall or in the middle of the striation. Electron-microscope investigation of the disturbances has not been as carefully related to cell age as was Green's investigation but the observations are generally in harmony with Green's conclusions.

DISCUSSION

X-ray diffraction analysis, chemical analysis, and infra-red spectrophotometry together, therefore, confirm that the skeletal material in the wall of *Nitella opaca* is a cellulose. This material, comprising about 17 per cent. of the wall by weight, yields on hydrolysis mannose and xylose as well as glucose, resembling in this respect the cellulose of many of the marine algae studied by Cronshaw, Myers, and Preston (1958). All examination procedures demonstrate the presence of amorphous incrusting substances and the chemi-

cal analysis presented in Table II shows that these include compounds of the polyuronide type. The diffuse appearance of the X-ray diagrams suggests that the cellulose crystallites are unusually narrow, which is well understandable since the microfibrils are only about 50 Å. wide.

The wall is lamellated and the high degree of swelling in thickness, coupled with the easy separation of the lamellae after extraction with ammonium oxalate, shows that the polyuronides contribute toward the binding together of the lamellae. The lamellae themselves may be stripped as coherent sheets even after such chemical treatments, presumably because the microfibrils, though tending toward a preferred orientation, show considerable scatter about this direction with some degree of entanglement. The microfibrils of an innermost wall lamella tend toward an orientation which is approximately transverse whereas those of an outermost wall lamella tend toward longitudinal orientation, a difference which is quite clear-cut and decisive. While transitions between the one orientation and the other in intermediate lamellae have not been observed, this is precisely the kind of evidence which led Roelofsen (1951) and Roelofsen and Houwink (1953) to their multinet-growth hypothesis for other cell types and in part confirms the conclusions reached by Green (1960) by more indirect methods. The high degree of angular dispersion of the microfibrils in the innermost lamella and their apparent entanglement shows, however, that if the structure of the outermost lamella has been produced by the longitudinal extension of a lamella initially resembling the innermost lamella, then the microfibrils must be free to slide over each other. This is a feature common, in greater or less degree, to all cell walls to which the multinet-growth hypothesis has been applied and it is common experience that carelessness during the stripping of a wall can lead to a pulling out of individual microfibrils from a lamella within which they have appeared to be hopelessly entangled. Multinet growth may well therefore be involved in the extension of this particular cell wall.

In considering both the mechanism of cellulose orientation and the changes in the orientation arising as a consequence of growth, note must of course be taken of the fact that the orientation of the microfibrils of the innermost—and therefore most recently deposited—layer is far from universally transverse. On the contrary, microfibrils can already be observed lying displaced at all azimuths from the transverse with a tendency, indeed, to favour the longitudinal as a second preferred orientation less marked, but no less certain, than the approximately transverse. It seems unlikely that this innermost lamella could already have been subjected to a longitudinal extension sufficient to cause a major reorientation, and the conclusion seems inescapable that the lamellae are laid down with this diversity of orientation. The structure recalls that already reported in the S1 layer of the secondary wall of a conifer tracheid (Frei *et al.*, 1957). It is not at the moment clear whether this is to be regarded as expressing an imperfection in a mechanism imposing predominantly transverse orientation or whether this wall represents a crossed fibrillar structure differing only in perfection from that shown by some other algae (Preston

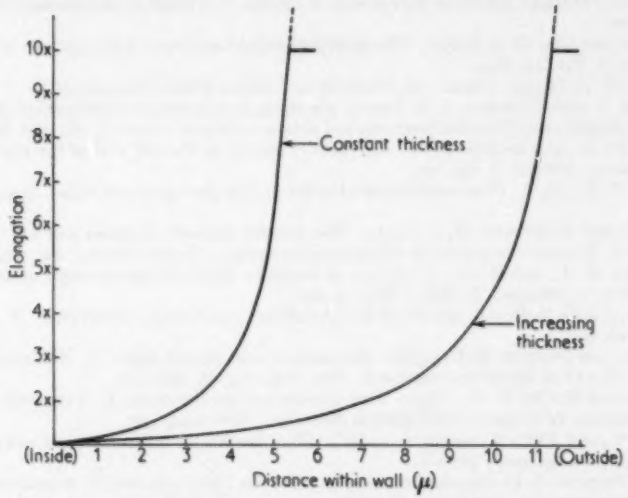
and Astbury, 1937; Astbury and Preston, 1940; Nicolai and Frey-Wyssling, 1938; Steward and Muhlethaler, 1953; Cronshaw and Preston, 1958; Frei and Preston, 1961).

Possible changes in orientation of the microfibrils as a cell elongates have been followed only through measurement of the m.e.p., i.e. by determining the net orientation throughout the wall thickness in cells of various lengths. The mutual correlations between cell length, m.e.p., and the direction of streaming in the cytoplasm appear to be rather complex. This makes it unlikely that the direction of the m.e.p. can causally be connected with cell length. Moreover, it can be shown that the process of multinet growth and extension need not produce major changes in the overall fibril distribution and, therefore, in the m.e.p.[1] In this type of growth the whole wall is considered, at any stage, to stretch passively and therefore becomes proportionally thinner. This reduction in thickness is counterbalanced, to a greater or less degree, by simultaneous apposition of new wall lamella on the inner face of the wall, which then comes in turn under stress. Suppose, therefore, a young cell, with a wall 6 μ thick, increases in length ten times. For the sake of simplicity imagine that first the cell, of length l, increases in length to $6/5{\cdot}5l$ and then lays down a new lamella 0·5 μ thick so that the wall remains 6 μ thick. By repetition of this process, a state can be reached at which the cell has become ten times as long while the wall has maintained its thickness of 6 μ. Though cells do not grow in this stepwise fashion the resultant elongations, after deposition, of the lamellae successively laid down will give a fair approximation to the truth. Fig. 6 shows the amount by which each lamella of the wall has extended, when a cell has increased in this way 10× in length for the condition of constant wall thickness outlined above and for the condition in which the wall increases in thickness by 0·5 μ at each step. In either case the outermost lamella, 0·6 μ thick, represents the original 6 μ-thick wall. Remembering that the original cell itself would already have extended, then the actual curve would extend steeply as shown by the dotted line. Now it is clear that in a cell at any stage of growth the distribution of strain through the wall will be much as exemplified in Text-fig. 6. Hence, after any subsequent elongation the fibril distribution (and the m.e.p.) will tend to be the same. This argument assumes that the newly deposited innermost lamella has, at each stage, the same structure, and assumes that the proportion of longitudinal to diametral extension is similar throughout growth (i.e. $\delta l/l = k\,\delta l/b$, where k is a constant). The fact that the birefringence remains almost constant during growth is experimental evidence in support of this suggestion.

It does not therefore seem probable, either on these theoretical grounds or on the basis of the results presented in this paper, that the observed correlation between cell length and wall structure can imply a direct causal connexion. The change in the m.e.p. must rather be associated with changes in the microfibrillar orientation in the successive innermost lamellae deposited

[1] This was pointed out to us independently by Dr. Barber of the Dominion Physical Laboratory, New Zealand.

as a cell grows. These changes must in turn be a consequence of some property of the cytoplasm which changes with cell length and the only property known so far to change is the direction of streaming. It could therefore be that the direction of streaming—which changes as a cell elongates—is involved in the orientation of microfibrils. Such a connexion can hardly be direct since



TEXT-FIG. 6. The distribution of strain at various points within a wall which, initially uniform, has extended overall by 10 times while either maintaining its thickness at 6 μ or increasing it. For further explanation, see text.

presumably the layer of cytoplasm nearest to the wall is stationary. If a connexion does exist, it is rather to be sought through an orientation of protein chains or chain aggregates in the stationary layer, the microfibrils being oriented at right angles to the protein chains. The variation in the angle α of Table IV could then be due to differing rates of cell elongation, relative to the amount of wall deposited, as a cell grows.

LITERATURE CITED

ASTBURY, W. T., and PRESTON, R. D. (1940). The structure of the cell wall in some species of the filamentous green alga *Cladophora*. *Proc. Roy. Soc.* B, **129**, 54.

BEAR, R. S., and FRENCH, D. (1941). Significance of X-ray diffraction patterns obtained from starch granules. *J. Amer. Chem. Soc.* **63**, 2298.

CORRENS, C. (1893). Zur Kenntnis der inneren Struktur einiger Algenmembranen. *Zimmermann's Beitr. Morph. und Physiol. der Pflanzenz.* **1**, 260.

CRONSHAW, J., MYERS, A., and PRESTON, R. D. (1958). A chemical and physical investigation of the cell walls of some marine algae. *Biochim. et Biophys. Acta*, **27**, 89.

—— and PRESTON, R. D. (1958). A re-examination of the fine structure of the walls of vesicles of the green alga *Valonia*. *Proc. Roy. Soc.* B, **148**, 137.

DEBSKI, B. (1898). Beobachtungen über Kernteilung bei *Chara fragilis*. *Jahrb. wiss. Bot.* **30**, 227.

FREI, EVA, and PRESTON, R. D. (1960). Unpublished.

—— —— (1961). *Proc. Roy. Soc.* B. To be published.

FREI, EVA, PRESTON, R. D., and RIPLEY, G. W. (1957). The fine structure of the walls of conifer tracheids. VI. Electron microscope investigations of sections. *J. Exp. Bot.* **8**, 139.

GREEN, P. B. (1954). Spiral growth pattern of the cell wall in *Nitella axillaris*. *Amer. J. Bot.* **41**, 403.

—— (1958). Structural characteristics of developing *Nitella* internodal cell walls. *J. Biophys. and Biochem. Cytology*, **4**, 505.

—— (1959). Wall structure and helical growth in *Nitella*. *Biochim. Biophys. Acta*, **36**, 536.

—— (1960). Multinet growth in the cell wall of *Nitella*. *J. of Biophys. and Biochem. Cytology*, **7**, 289.

—— and CHAPMAN, G. B. (1955). The development and structure of the cell wall in *Nitella*. *Amer. J. Bot.* **42**, 685.

HERMANS, P. H. (1949). *Physics and Chemistry of Cellulose Fibres*. Elsevier, N.Y.

HODGE, A. J., and WARDROP, A. B. (1950). An electron microscopic investigation of the cell wall organization of conifer tracheids and conifer cambium. *Austr. J. Sci. Res.* B, **3**, 265.

JERMYN, M. A., and ISHERWOOD, F. A. (1956). Changes in the cell wall of the pear during ripening. *Biochem. J.* **64**, 123.

KREGER, D. R. (1957). New orientations of cellulose I in *Spirogyra* cell walls. *Nature*, **180**, 914.

MANN, J., and MARRINAN, H. J. (1956). The reaction between cellulose and heavy water. Part I. A qualitative study by infra-red spectroscopy. *Trans. Faraday Soc.* **52.2**, 481.

MARRINAN, H. J., and MANN, J. (1954). A study by infra-red spectroscopy of hydrogen bonding in cellulose. *J. Appl. Chem.* **4**, 204.

—— —— (1956). Infra-red spectra of the crystalline modifications of cellulose. *J. Polymer Sci.* **21**, 301.

MYERS, A., and PRESTON, R. D. (1959). Fine structure in the red algae. II. The structure of the cell wall of *Rhodymenia palmata*. *Proc. Roy. Soc.* B, **150**, 447.

—— —— and RIPLEY, G. W. (1956). Fine structure of the red algae. I. X-ray and electron microscope investigation of *Griffithsia flosculosa*. Ibid. **144**, 450.

NICOLAI, E., and FREY-WYSSLING, A. (1938). Über den Feinbau der Zellwand von *Chaetomorpha*. *Protoplasma*, **30**, 401.

—— and PRESTON, R. D. (1952). Cell wall studies in the Chlorophyceae, I. A general survey of submicroscopic structure in filamentous species. *Proc. Roy. Soc.* B, **140**, 244.

PRESTON, R. D. (1951). Fibrillar units in the structure of native cellulose. *Faraday Soc. Disc.* **11**, 165.

—— and ASTBURY, W. T. (1937). The structure of the wall of the green alga *Valonia ventricosa*. *Proc. Roy. Soc.* B, **122**, 76.

—— and CRONSHAW, J. (1958). Constitution of the fibrillar and non-fibrillar components of the walls of *Valonia ventricosa*. *Nature*, **181**, 248.

—— NICOLAI, M. F. E., REED, R., and MILLARD, A. (1949). An electron microscope study of cellulose in the wall of *Valonia ventricosa*. *Nature*, **162**, 665.

—— and SINGH, K. (1950). Fine structure of bamboo fibres. I. Optical properties and X-ray data. *J. Exp. Bot.* **1**, 214.

PROBINE, M. C. (1959). *Molecular structure and mechanical properties of plant cell walls in relation to growth*. Ph.D. Thesis, Leeds.

—— and PRESTON, R. D. (1958). Protoplasmic streaming and wall structure in *Nitella*. *Nature*, **182**, 1657.

RÅNBY, B. G. (1952). Physico-chemical investigations on animal cellulose. *Arkiv. för Kemi*. **4**, 241.

ROELOFSEN, P. A. (1951). Orientation of cellulose fibrils in the cell wall of growing cotton hairs. *Biochim. et Biophys. Acta*, **7**, 43.

—— and HOUWINK, A. L. (1953). Architecture and growth of the primary cell wall in some plant hairs and in the *Phycomyces* sporangiophore. *Acte Botan. Néerl.* **2**, 218.

SCHWENDENER, S. (1874). *Das mechanische Prinzip im anatomischen Bau der Monocotyleen*. Leipzig.

SEN, M. K., and WOODS, H. J. (1948). X-ray investigation of the structure of jute. *Nature*, **161**, 768.

STEWARD, F. C., and MUHLETHALER, K. (1953). The structure and development of the cell wall in the Valoniaceae. *Ann. Bot.* (*Lond.*), N.S. **17**, 295.

VOTAVA, A. (1914). Beiträge zur Kenntnis der Inhaltskörper und der Membran der Characeen. *Oester. Bot. Zeitschr.* **64**, 442.

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PLATES

FIG. 1. X-ray sector diagram of *Cladophora* cellulose (top right and bottom left), and *Nitella opaca* wall after treatment with 2% H_2SO_4 (top left and bottom right). Note the correspondence between the rings in the two diagrams.

FIG. 2. X-ray diagram of a stack of aligned strips of untreated *Nitella opaca* wall: beam normal to wall surface, cell axis parallel to longer edge of page. The white shadow is due to the support of the front beam-stop. Note that the rings are most intense along the meridian.

FIG. 3. As in Fig. 2, but beam parallel to wall surface and perpendicular to the long axis of the cell.

FIG. 4. Photomicrograph of a single wall of *Nitella opaca* between crossed polaroids, showing a striation passing from lower left to upper right. An elliptic compensator was inserted in the light path and displaced slightly from the position in which the wall was extinguished.

FIG. 5. Composite photograph of a striation between crossed polaroids at various settings of an elliptic compensator. Compensator settings shown at the side of each photographic strip. For further explanation, see text.

FIG. 6. Photomicrograph of torn piece of wall between crossed polaroids showing about 5 lamella, and one striation passing obliquely upwards.

FIG. 7. Electron micrograph of a wall lamella shadowed Pd-Au, magnification 120,000×.

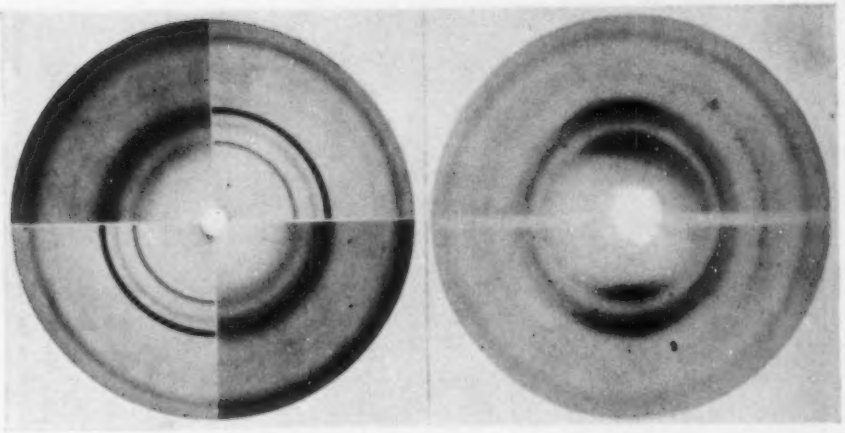
FIG. 8. Electron micrograph of the innermost wall lamella of a young internodal cell; shadowed Pd-Au; magnification 27,900×. Axis of cell parallel (approx.) to longer edge of page. Note that most microfibrils run almost transversely though many lie at other azimuths. The polar curve drawn on the lower right gives a measure of the angular distribution of the microfibrils, the length of line joining any point on the curve to the centre being proportional to the number of microfibrils lying in the direction of the line. There is therefore a marked tendency toward a crossed microfibrillar structure.

FIG. 9. Electron micrograph of a wall lamella in the area between two striations; shadowed Pd-Au; magnification 15,000×. Note the areas of disturbed orientation.

FIG. 10. Electron micrograph of a wall lamella of unknown location in the wall of an internodal cell; shadowed Pd-Au; magnification 27,900×. Axis of cell parallel (approx.) to longer edge of page. Note clear signs of a tendency toward a crossed fibrillar structure.

FIG. 11. As in Fig. 10, except that the lamella is the outermost coherent lamella of a young cell. Note the tendency toward axial orientation of microfibrils.

FIG. 12. Electron micrograph of a wall lamella at a striation; shadowed Pd-Au; magnification 7,200×.



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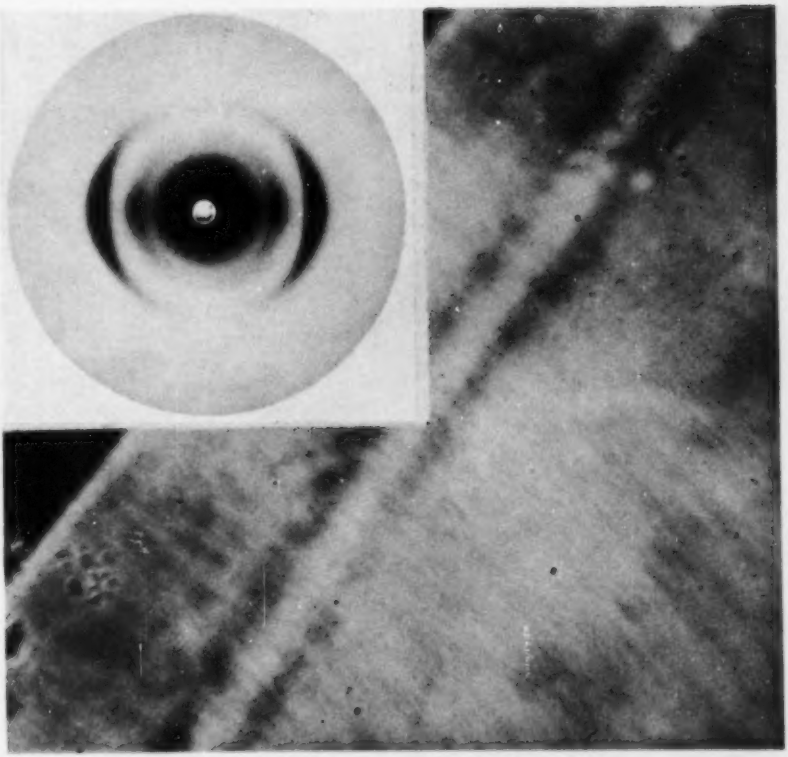

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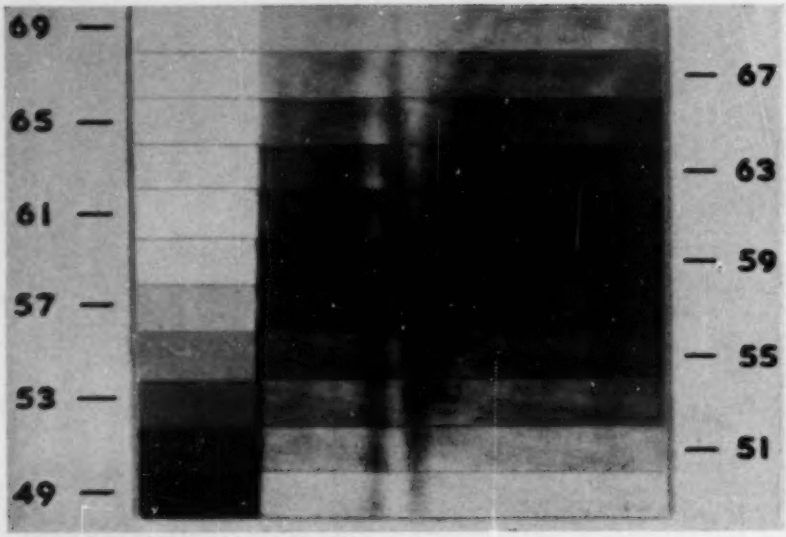
M. C. PROBINE AND R. D. PRESTON—PLATE I



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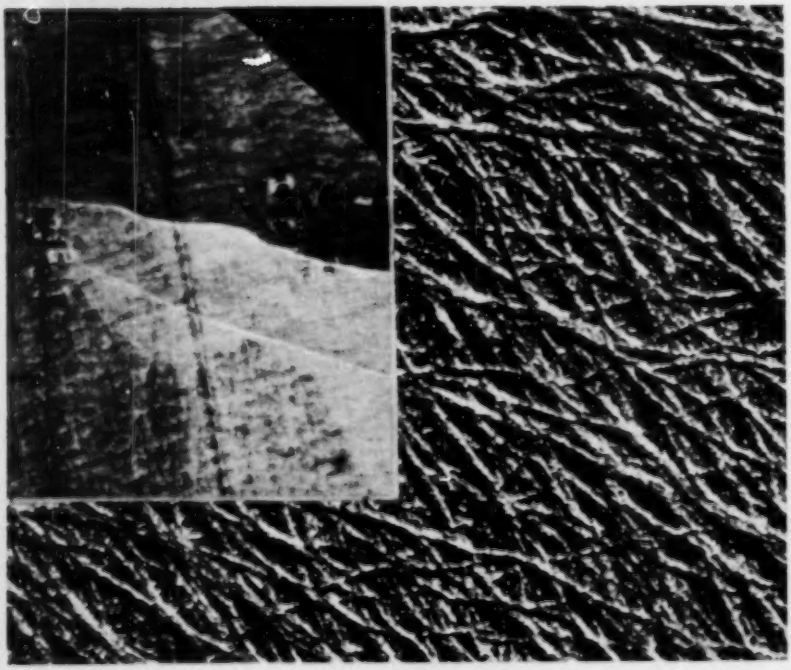

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M. C. PROBINE AND R. D. PRESTON—PLATE II

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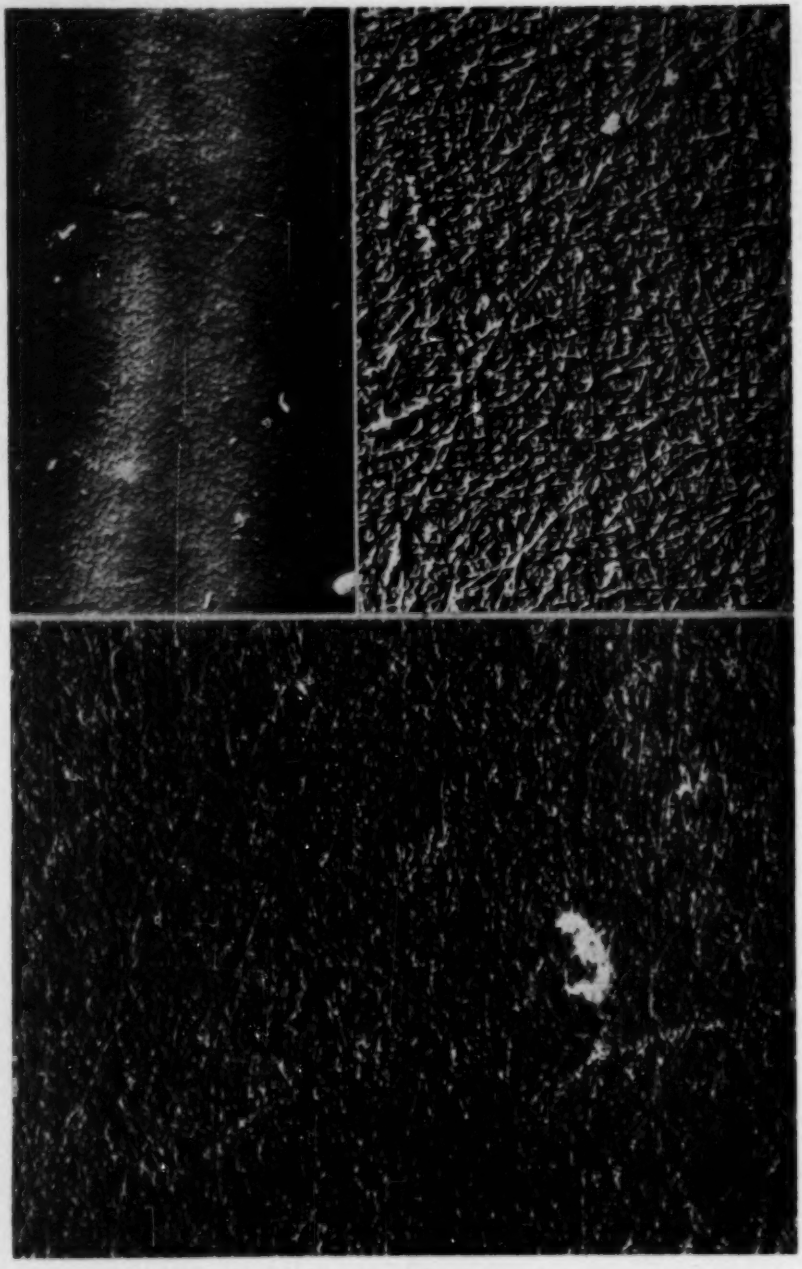
M. C. PROBINE AND R. D. PRESTON—PLATE III



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M. C. PROBINE AND R. D. PRESTON—PLATE IV

Duration of the Mitotic Cycle in a Meristem

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WITH TWO FIGURES IN THE TEXT

Received 23 July 1960

SUMMARY

The length of the mitotic cycle and the time spent in mitosis are calculated for six regions of the apical meristem of roots of *Zea*. The two methods used are the measurement of the rates of labelling nuclei with radioactive thymidine and the rates of accumulation of metaphases in roots grown in colchicine. The mitotic cycle lasts from 12 hours in the cap initials to about 200 hours in the quiescent centre of the same roots. Some points about the organization of apices are discussed in the light of these estimates.

INTRODUCTION

MERISTEMS of roots and shoots are complexly organized structures. Their cells synthesize new protoplasm, grow, and divide. The planes of division differ in different parts of the meristem and it is clear that the rates of division must also differ. I have already shown that rates of synthesis of desoxyribose nucleic acid (DNA), which are related to rates of division, do differ in root meristems (Clowes, 1956*a*, 1956*b*, 1958). In particular, a central part of the meristem, called the quiescent centre, has a rate of synthesis of DNA far below that of the rest of the meristem. This quiescent centre probably exists in all but the smallest roots and those with single apical cells. French workers have also shown that the summit of shoot meristems is less active than the flanks. They formerly believed that it was completely inactive except when the meristem is producing flowers. This is unlikely, but it illustrates the difficulty of this kind of work. The easiest way of comparing rates of division is to count the percentage of nuclei in mitosis (the Mitotic Index) and this is what the French workers did. But the Mitotic Index is merely a relation between mitotic duration and interphase duration and can be misleading when used this way, especially where the rates of division are low, because the time a nucleus spends in mitosis is a small and variable fraction of the total mitotic cycle. The Mitotic Index gives no indication of the length of the mitotic cycle because no measurement of time enters into its calculation.

Valid methods of measuring the rates of mitosis are available now. They have been used only on whole meristems to give average values for the duration of the mitotic cycle and its various phases. This paper describes adaptations of two of these methods to give time parameters for different parts of the meristem, for it is these, rather than average values, which interest students of development. The two methods used are the accumulation of metaphases by colchicine treatment and the labelling of nuclei by feeding the meristem with a radioactive precursor of DNA. The meristems used are those of the

primary roots of seedlings of *Zea mays* ('Golden Bantam'). Most of the previous measurements of the length of the mitotic cycle have been made on root apices of *Vicia faba*. For the present work *Zea* was chosen in spite of its smaller size and smaller chromosomes because it is easier to indentify the site of the quiescent centre and the boundaries of the histogens in grass roots.

EXPERIMENTAL METHODS AND RESULTS

1. *The Timing of DNA Synthesis*

This method of determining the time parameters of a meristem has been used by Howard and Pelc (1951, 1953) for root apices of *Vicia faba* to obtain an average value for the duration of the mitotic cycle (T) for the whole meristem. The technique is to feed the roots with a radioactively labelled precursor of DNA for varying lengths of time and to count the proportion of labelled nuclei in both interphase and mitosis. In the original method ^{32}P-labelled phosphate was used by Howard and Pelc, and, after feeding, the RNA was removed by hydrolysis. In the present experiments tritium-labelled thymidine is used. Seedlings of *Zea* are transferred to thymidine solution (250–500μ C./l.) when they are 20 mm. long and the solution, at 17° C., is aerated and kept dark. Samples of roots are fixed after 4, 8, 16, 32, 48, and 72 hours in the solution. The apices are sectioned transversely or longitudinally and autoradiographs are prepared on high-resolution stripping film. After exposure and development the sections are stained through the film or they may be stained before applying the film.

The results are set out in Table I and Fig. 1.

It will be seen that, at first, nuclei in interphase become labelled more quickly than nuclei in mitosis. This is because DNA synthesis occurs at some stage between early and late interphase and those cells which are dividing at the beginning of the treatment have to wait until their nuclei enter interphase and pass into this synthetic stage before they take up the thymidine. Although the proportion of cells in mitosis which are labelled starts low it rises faster than the proportion of interphases which are labelled. This is partly due to the fact that some of the cells in some of the regions of the meristem may not divide at all and therefore do not synthesize DNA and partly because a proportion of interphase nuclei will be in late interphase and therefore just out of the synthetic part of the mitotic cycle at the start of the experiment. These nuclei will pass unlabelled into mitosis and each will contribute two unlabelled early interphase nuclei as a result of mitosis.

It will also be seen that practically all the cells of the central stele, endodermis, and cap initials are labelled by 32 hours from the beginning of feeding, but only 50 per cent. of the cortical cells around the quiescent centre and only 7 per cent. of the cells of the quiescent centre itself are labelled. At 72 hours still only 28 per cent. of the cells of the quiescent centre are labelled. This indicates either that the rate of mitosis is very low or that few of the cells are preparing for division (or that both these conditions obtain).

TABLE I

Cells labelled after feeding with thymidine-^3H

Region of apex	No. of roots counted	Duration of feeding (hours)	Labelled cells in mitosis (%)	Labelled cells in interphase (%)	Mitotic Index (%)
Quiescent	16	4	0	3·9±1·3	1·6
centre	18	8	0	4·8±0·9	1·3
	18	16	0	6·8±0·8	2·2
	18	32	20·7±8·2	7·5±1·2	4·0
	16	48	41·7±9·5	24·2±3·6	4·0
	20	72	66·7±5·1	27·8±4·2	3·9
Cortex around	8	4	5·6±2·5	39·9±3·6	3·8
quiescent	9	8	8·6±3·5	43·6±2·6	2·4
centre	9	16	31·3±4·1	52·4±2·2	7·5
	9	32	62·6±5·6	53·8±2·3	10·2
	8	48	67·8±11·5	50·0±9·1	12·3
	10	72	77·8±9·2	78·9±2·9	6·9
Cap initials	8	4	74·3±7·7	79·9±5·7	13·9
	8	8	79·2±6·3	92·8±1·3	8·8
	10	16	94·8±3·4	98·2±0·4	12·8
	11	32	91·4±6·1	97·5±0·9	11·7
Central stele	8	4	39·6±5·2	55·3±6·6	14·5
just above	8	8	80·6±6·4	77·3±4·0	12·9
quiescent	8	16	95·4±5·0	88·2±2·7	11·5
centre	9	32	96·0±1·6	91·7±1·4	11·6
	8	48	93·8±4·1	93·2±1·5	10·5
Central stele	8	4	6·0±3·2	38·8±3·2	19·1
200 μ from	9	8	13·4±3·7	49·5±3·5	20·5
quiescent	9	16	36·2±4·3	65·1±3·7	24·6
centre	9	32	89·0±4·1	88·8±2·4	22·1
	7	48	91·7±4·0	96·0±1·8	16·4
Endodermis	8	4	14·3±5·7	39·9±3·7	17·8
200 μ from	9	8	24·7±4·9	59·3±3·1	16·3
quiescent	9	16	58·9±7·3	72·3±4·9	16·9
centre	9	32	93·2±3·2	92·6±1·9	20·6
	7	48	98·3±2·0	97·3±0·9	14·4

Standard errors are given for the percentages plotted in Fig. 1.

There are signs that, after 72 hours, the roots are suffering from radiation damage and I have already shown that, when this happens after X-irradiation, cells of the quiescent centre may synthesize DNA and enter mitosis when other cells of the meristem, more sensitive to radiation, stop dividing (Clowes, 1959*a*). The fact that the Mitotic Index rises in the quiescent centre after long exposures to thymidine and falls in the other parts of the apex suggests that the β-radiation is beginning to have the same effect as the X-rays of the earlier experiment. Not only are meristematic cells more vulnerable to radiation than non-meristematic cells, but, in the present experiments, the meri-

stematic nuclei will also have taken in more tritium atoms than the quiescent nuclei. The figure of 28 per cent. labelled interphase cells in the quiescent centre may therefore be swollen by radiation damage to the rest of the meristem.

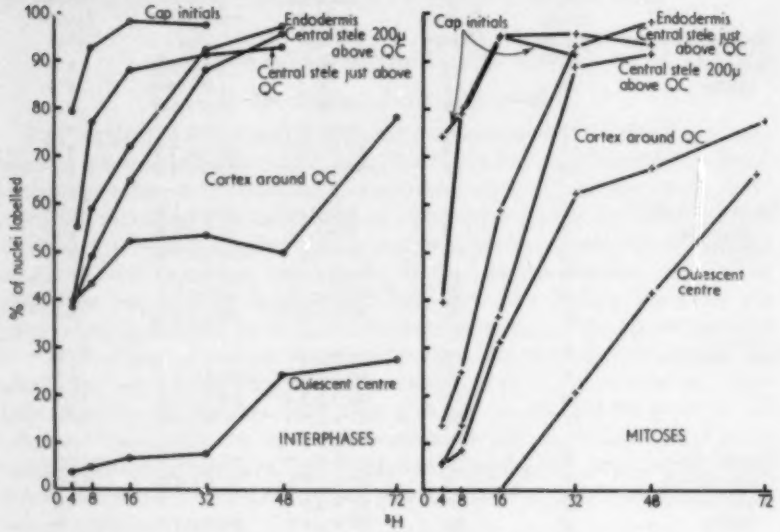

FIG. 1. Percentage of nuclei labelled after feeding roots of *Zea mays* with thymidine-^{3}H. Standard errors are given in Table I.

The time required to reach the maximum number of labelled interphase nuclei gives a value for the duration of the whole mitotic cycle (T). For the cap initials T appears to be about 12 hours; for the central stele just above the quiescent centre T is about 25 hours; for the central stele and endodermis at 200μ from the quiescent centre T is about 40 hours; for the cortex around the quiescent centre T is longer than 48 hours. No value for the duration of the mitotic cycle in the quiescent centre can be obtained from the present data and we have seen that it would be useless to extend the experiment beyond 72 hours. If the increase in the number of cells is exponential, the Mitotic Index is approximately equal to $(\tau/T)\log_e 2$ where τ is the duration of mitosis (Hoffman, 1949). Using the Mitotic Index measured at 4 hours, τ is 2·4 hours for the cap initials, 5·2 hours for the central stele just above the quiescent centre, 11·1 hours for the central stele, and 10·3 hours for the endodermis at 200μ from the quiescent centre, and longer than 2·6 hours for the cortex around the quiescent centre.

Two sources of error arise in the actual counts in this method. One is due to the difficulty sometimes experienced in deciding whether a nucleus is in mitosis or interphase when it is covered by an autoradiographic film. The

second is the difficulty of deciding whether a nucleus is labelled or not when the number of silver grains over a nucleus is nearly the same as the background count. The use of tritiated thymidine reduces errors from this cause to a minimum because of the low energy of the β-particles and the cells' restricted use of thymidine.

2. *Accumulation of metaphases*

The principle of this technique is that colchicine frustrates anaphase, but does not affect the rate of entry of cells into mitosis. If, therefore, meristems are treated with colchicine for various short periods the rate of entry into mitosis can be measured and hence the average duration of the mitotic cycle may be calculated. D'Amato and Avanzi (1948) have pointed out that prolonged treatment with colchicine leads to different degrees of polyploidy in root meristems. In particular, part of the apex remains diploid and it is now clear that this part is the quiescent centre, whose cells remain diploid because they never enter mitosis or do so very slowly.

The technique used here is adapted for use on specific parts of meristems from that used by Evans, Neary, and Tonkinson (1957) and Evans and Savage (1959) for whole meristems. Seedlings of *Zea* are taken when their roots are 20 mm. long and transferred to a vessel so that their roots dip into water in the dark. The water is aerated. After 16 hours the water is replaced by a 0·05 per cent. solution of colchicine. Batches of roots are fixed after 0, 2, 4, 8, 12, and 24 hours of immersion in colchicine, embedded in paraffin, and sectioned. An eyepiece graticule is useful for counting the nuclei and both longitudinal and transverse sections are counted. Sections are not so easy to use as squashes, especially in identifying the phases of mitosis, but they are essential for delimiting the regions of the meristem. The parts of the meristem counted are the same as those used in the previous experiment. Some meristems have their cell division partially synchronous, but previous observations have shown that roots of *Zea* grown under these conditions have no variation in Mitotic Index at different times of fixation.

The counts are given in Table II and, for metaphases, in Fig. 2. The Mitotic Index rises initially with increasing length of colchicine treatment and then falls between 12 and 24 hours after the beginning of the treatment. The rise is due largely to an increase in the number of cells at metaphase. The number of late stages of mitosis falls owing to the blocking of the process at metaphase; but it does not always fall to zero, because some cells may be slow to enter interphase or may even escape from metaphase to anaphase. The fall in the number of metaphases at the end of the colchicine treatment is due to a reduction in the number of cells entering mitosis and the escape of cells from metaphase to polypoid interphase. In calculating the parameters of the mitotic cycle from the rate of entry into metaphase therefore only the first part of the treatment may be used, except that Evans, Neary, and Tonkinson (1957) have shown that it is wise to neglect the first hour of treatment because different

TABLE II

Cells in stages of the mitotic cycle after treatment with colchicine

Region of apex	No. of roots counted	Duration of treatment (hrs.)	Prophases (%)	Metaphases (%)	Anaphase and Telophase (%)	Interphase %	Mitotic Index %
Quiescent centre	8	0	1·0	0·5±0·3	0·4	98·1	1·9
	8	2	0·3	1·3±0·3	0·6	97·8	2·2
	10	4	1·6	2·1±0·6	0·0	96·4	3·7
	8	8	4·1	4·7±0·8	0·0	91·3	8·8
	9	12	3·2	5·6±2·5	0·0	91·3	8·8
	9	24	3·0	3·4±1·1	0·0	93·7	6·4
Cortex around quiescent centre	10	0	3·2	2·6±0·6	1·1	93·2	6·9
	8	2	2·0	4·0±0·6	1·3	92·7	7·3
	10	4	5·7	6·6±1·0	0·3	87·6	12·6
	8	8	5·1	11·0±1·6	0·5	83·4	16·6
	9	12	5·7	15·3±2·2	0·2	78·9	21·2
	9	24	5·0	10·7±1·5	0·0	84·4	15·7
Cap initials	6	0	5·7	5·4±0·9	4·6	84·3	15·7
	7	2	7·2	6·7±1·4	4·6	81·7	18·3
	8	4	11·2	20·0±2·0	0·2	68·7	31·3
	8	8	6·7	23·3±4·4	0·3	69·7	30·3
	6	12	10·7	22·1±1·8	0·3	66·8	33·2
	8	24	6·6	17·1±1·3	1·0	75·3	24·7
Central stele just above quiescent centre	10	0	4·9	3·2±0·5	2·6	89·3	10·8
	8	2	6·8	6·5±1·1	2·3	84·5	15·6
	10	4	8·8	11·8±1·2	0·2	79·2	20·8
	8	8	10·1	17·1±1·4	0·6	72·2	27·8
	9	12	9·4	24·9±2·2	0·0	65·8	34·3
	9	24	8·4	18·8±1·8	0·0	72·8	27·2
Central stele 200 μ from quiescent centre	6	0	7·1	5·2±0·8	4·5	83·3	16·7
	8	2	6·5	8·3±1·1	1·8	83·5	16·5
	8	4	7·8	13·6±1·8	0·2	78·4	21·6
	8	8	7·3	13·7±1·5	0·5	78·8	21·2
	8	12	9·7	13·3±1·6	0·8	76·3	23·7
	8	24	9·1	12·5±0·8	0·0	78·6	21·4
Endodermis 200μ from quiescent centre	6	0	9·8	4·9±0·7	3·0	82·3	17·7
	8	2	8·3	9·1±1·0	0·9	81·7	18·3
	8	4	7·8	13·6±1·5	0·2	78·4	21·6
	8	8	7·7	17·5±2·6	0·0	74·8	25·2
	8	12	7·7	10·5±3·7	0·8	72·2	27·8
	8	24	8·9	15·3±2·2	0·2	75·7	24·3

Standard errors are given for the metaphases plotted in Fig. 2.

concentrations of colchicine disrupt already formed mitotic spindles with different efficiencies at the beginning of the treatment.

In the following calculation the method of Evans, Neary, and Tonkinson is used except that no correction is made for any cells which may escape from metaphase to anaphase and beyond. In the calculation it is assumed that all the cells are meristematic except in the quiescent centre where correction factors are inserted. This assumption that all or nearly all the cells divide has been shown to be true in the previous experiment for the cap initials, endodermis, and central stele. It may also be true for the cortex around the quies-

cent centre. For the quiescent centre it is impossible to tell from the previous experiment whether all the cells are meristematic or not and the calculation is modified by correction factors for both 70 and 90 per cent. inert cells for the reasons given above.

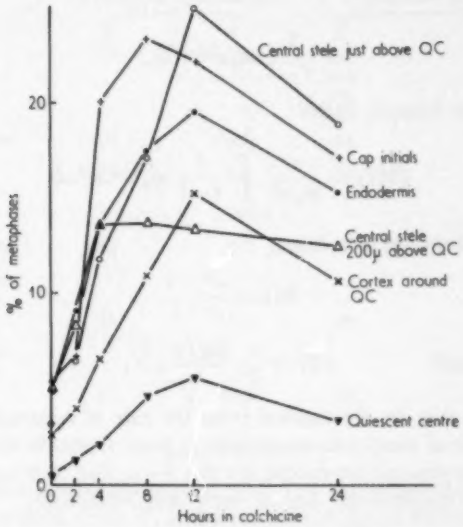

FIG. 2. Percentage of nuclei in metaphase after treating roots of *Zea mays* with colchicine. Standard errors are given in Table II.

The increase in the number of cells is assumed to be exponential. Hence

$$m = m_0 e^{\lambda t}$$

where m_0 is the number of meristematic cells at time t, and λ is a constant. There is some evidence that the growth of cell number in root apices is not exponential (Erickson and Sax, 1956), but the assumption that it is is probably justified in the present work.

If T is the duration of the whole mitotic cycle and l is the fraction of the cells which do not divide, m_0 cells will become $(2-l)m_0$ cells between $t = 0$ and $t = T$.

Therefore $$e^{\lambda T} = 2-l$$

and $$\lambda = \frac{\log_e(2-l)}{T}.$$

A dividing cell contributes $(1-l)$ extra meristematic cells to the population; therefore the number of cells dividing in unit time equals the rate of increase of the population.

i.e. $$\frac{1}{1-l}.\frac{dm}{dt} = \frac{\lambda}{1-l}.m_0 e^{\lambda t}.$$

At a time, t, the number of cells which reach the end of mitosis after a further period between s and $s+ds$ equals the number of cells dividing per unit time at a time, $t+s$,

i.e. $$\frac{\lambda}{1-l}.m_0 e^{\lambda(s+t)}\,ds.$$

Therefore the Mitotic Index

$$(\mathrm{MI}) = \frac{1}{m_0 e^{\lambda t}} \int_{s=0}^{s=\tau} \frac{\lambda}{1-l}.m_0 e^{\lambda s}.e^{\lambda t}.ds$$

where τ is the duration of mitosis.

Therefore $$\mathrm{MI} = \frac{e^{\lambda\tau}-1}{1-l}$$

and if τ/T is small $$\mathrm{MI} \simeq \frac{\tau}{T}.\frac{\log_e(2-l)}{1-l}.$$

The value of λ may be determined from the rate of accumulation of metaphases. The rate of entry into metaphase at time, t, equals the rate at which cells would have entered interphase at time, $t+s_2$, had they not been stopped at metaphase. Therefore the rate of entry into metaphase,

$$\frac{dM}{dt} \text{ equals } \frac{\lambda}{1-l}.m_0 e^{\lambda(t+s_2)}$$

where M is the number of cells at metaphase at time, t.
The slope of the curves in Fig. 2 between 2 hours and 4 hours is

$$\frac{e^{\lambda s_2}}{1-l}.\lambda(1+\lambda).$$

s_2 is a constant marking the boundary between prophase and metaphase in untreated roots or in roots treated with colchicine. The ratio of the number of cells in metaphase and later stages of mitosis to the total number of cells equals $e^{\lambda s_2}-1/1-l$ and this may be found by counting cells in normal roots. This equation and that for the slope of the curve of metaphases provide a quadratic equation from which λ may be calculated and hence T and τ.

Taking the accumulation of metaphases between 2 and 4 hours and assuming $l=0$, T is 12 hours for the cap initials, 28 hours for the stele just above the quiescent centre, and 29 hours at 200μ from the quiescent centre, 35 hours for the endodermis at 200μ from the quiescent centre, and 55 hours for the cortex around the quiescent centre. For the quiescent centre itself, T is 174 hours if it is assumed that all the cells divide, 220 hours if 30 per cent. divide, and 239 hours if 10 per cent. divide. Values for τ based on these values of T and the Mitotic Indices at 0 hours are given in Table III.

TABLE III

Duration of the mitotic cycle (T) and mitosis (τ) calculated from rates of thymidine labelling and metaphase accumulation

	Thymidine labelling		Metaphase accumulation	
	T	τ	T	τ
Quiescent centre	—	—	174	4·8 (l=0)
			220	4·8 (l=0·7)
			239	4·5 (l=0·9
Cortex around quiescent centre	>48	>2·6	55	5·5
Cap initials	12	2·4	12	2·7
Central stele just above quiescent centre	25	5·2	28	4·4
Central stele 200μ from quiescent centre	40	11·1	29	7·0
Endodermis 200μ from quiescent centre	40	10·3	35	8·9

DISCUSSION

The two methods reported here provide two independent estimates of the duration of the mitotic cycle, T, and therefore the rate of division, for different parts of the meristem. The estimates of other workers are mostly averages for the whole meristem. For *Vicia faba*, Gray and Scholes's (1951) value for T is 25 or 19 hours according to how much of the root tip is included in the average. Howard and Pelc's estimate is 30 hours at 19° C. Evans and Savage's estimate is 260 hours at 3° C., 64 hours at 10° C., 26 hours at 19° C., and 23 hours at 25° C. For *Pisum sativum*, Brown's (1951) estimate is 25 hours at 15° C. and 14 hours at 30° C. Hejnowicz (1959) provides estimates for T at various levels of the root apices of *Triticum* grown at 18° C. His method is an analysis of the numbers of cells and the rates of growth and provides values of between 20 and 60 hours. At the level of the pole of the stele T is 25 hours in the epidermis. With increasing distance from the tip the value falls to 21 hours at 0·15 mm. and then rises to 60 hours at 0·9 mm. The values for the other tissues follow a similar pattern. Hejnowicz does not give values specifically for the quiescent centre, but he believes that his results are consistent with its existence. The results of the present work show a wider variation in the value of T than Hejnowicz has found, but his analysis is not designed to show the maximum variation and his subject, *Triticum*, having smaller roots, probably has less variation t an *Zea*. Erickson and Sax (1956) find that the maximum relative elemental rate of cell formation of 0·16 per hour occurs at 1·25 mm. from the tip in *Zea*, i.e. probably 700μ above the quiescent centre. The present observations do not extend so far nor do the methods of Erickson and Sax enable them

to pick out such small regions of the meristem as the group of cap initials which has the highest rate of division measured here.

Hejnowicz holds that the lengths of the various phases of mitosis do not vary much from one part of the meristem to another. His results are again based on growth rates and the time from 'prometaphase' to 'early telophase' varies only from 0·5 to 0·8 hours. Prophase is the longest stage of mitosis and Hejnowicz allots only 13 per cent. of τ to 'prometaphase'. His method of counting mitotic figures therefore differs from mine and those of other workers. This, no doubt, accounts for his very low value for τ and may also partially explain why there is so little variation in τ in his results.

Most of the previous work on meristems has been based on the assumption that all the cells behaved similarly. Clearly this is not so. The Mitotic Index varies from about 2 per cent. in the quiescent centre to about 20 per cent. Average values of about 10 per cent. for whole meristems have been found by previous workers. In the past, the Mitotic Index has commonly been taken to indicate the rate of division. The fallacy of this has been pointed out before (Clowes, 1960) and is clearly demonstrated in the present calculations. Not only is τ a variable fraction of T, but it is a small and variable parameter. The cap initials have the highest rate of division among the regions investigated here, once in 12 hours, but their Mitotic Index is smaller than that of the stele and endodermis at 200μ which have only a third of their rate of division.

The time intervals between the observations are too large in the labelling experiments to see whether all the parts of the mitotic cycle are extended equally where T is long. However, τ is not extended in proportion to T so that it may well be that the presynthetic (G_1) and postsynthetic (G_2) stages of interphase are not extended equally. A differently designed timing experiment will have to be used to determine that.

These results give a quantitative estimate for what is meant by quiescence. Over 70 per cent. of the cells of the quiescent centre have not prepared for division in 72 hours in these seedlings of *Zea* and the mitotic cycle is extended to possibly 20 times its duration in the neighbouring cells. They confirm my previous views about the organization of root meristems (Clowes, 1959*b*, 1960) which are still misinterpreted by Guttenberg (1960), the proponent of an opposing theory. In particular they justify the siting of the initials of a root over the surface of the quiescent centre and the rather ephemeral nature ascribed to these initials. The methods used here will now be applied to shoot apices about whose organization a great deal less is known.

A point which does not emerge from any of the previous work is that the cells immediately proximal and immediately distal to the quiescent centre are the ones with the highest rate of division in the distal part of the apex. This may be significant in explaining the quiescent centre. The rapidly dividing cells of the central stele and cap initials may starve the quiescent centre of nutrients, or the low rate of synthesis in the quiescent centre may enable the central stele and the cap initials to synthesize more rapidly than if they were adjacent to actively meristematic cells. This possibility does not preclude a

theory which depends upon oxygen or auxin controlling DNA synthesis (Clowes, 1961). The difficulty of all these theories is how to explain the sharpness of the boundary between the quiescent centre and the cap initials.

ACKNOWLEDGEMENTS

I am indebted to Dr. H. J. Evans for discussing this paper with me and to the Royal Society for financial help.

LITERATURE CITED

BROWN, R. (1951). The effects of temperature on the durations of the different stages of cell division in the root-tip. *J. exp. Bot.* **2**, 96–110.

CLOWES, F. A. L. (1956*a*). Nucleic acids in root apical meristems of *Zea*. *New Phytol.* **55**, 29–35.

—— (1956*b*). Localization of nucleic acid synthesis in root meristems. *J. exp. Bot.* **7**, 307–12.

—— (1958). Development of quiescent centres in root meristems. *New Phytol.* **57**, 85–88.

—— (1959*a*). Reorganization of root apices after irradiation. *Ann. Bot.* N.S. **23**, 205–10.

—— (1959*b*). The root apical meristem and its cellular organizaton. *Proc. IX Internat. bot. Congress*, **2** 76–77.

—— (1961). *Apical meristems*. Oxford, Blackwell Scientific Publications (in the press).

—— (1961). Cell development and differentiation in root apices. *Proc. IX Internat. bot. Congress*, **3** (in the press).

D'AMATO, F., and AVANZI, M. G. (1948). Reazioni di natura auxinica ed effetti rizogeni in *Allium cepa*. *Nuovo G. bot. ital.* N.S. **56**, 161–213.

ERICKSON, R. O., and SAX, K. B. (1956). Rates of cell division and cell elongation in the growth of the primary root of *Zea mays*. *Proc. Amer. phil. Soc.* **100**, 499–514.

EVANS, H. J., NEARY, G. J., and TONKINSON, S. M. (1957). The use of colchicine as an indicator of mitotic rate in broad bean root meristems. *J. Genet.* **55**, 487–502.

—— and SAVAGE, J. R. K. (1959). The effect of temperature on mitosis and on the action of colchicine in root meristem cells of *Vicia faba*. *Exp. Cell Res.* **18**, 51–61.

GRAY, L. H., and SCHOLES, M. E. (1951). The effect of ionizing radiations on the broad bean root. Part VIII. Growth rate studies and histological analyses. *Brit. J. Radiol.* N.S. **24**, 82–92, 176–80, 228–36, 285–91, 348–52.

GUTTENBERG, H. VON (1960). *Grundzüge der Histogenese höherer Pflanzen. I. Die Angiospermen*, Berlin, Gebrüder Borntraeger.

HEJNOWICZ, Z. (1959). Growth and cell division in the apical meristem of wheat roots. *Physiol. Plant.* **12**, 124–38.

HOFFMAN, J. G. (1949). Theory of the mitotic index and its application to tissue growth measurement. *Bull. math. Biophys.* **11**, 139–44.

HOWARD, A., and PELC, S. R. (1951). Synthesis of deoxyribose nucleic acid and nuclear incorporation of ^{35}S as shown by autoradiographs. *Isotopes in Biochemistry* (C.I.B.A.), 138–48.

—— —— (1953). Synthesis of desoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. *Heredity*, **6**, Suppl. 261–73.

The Development of the Graft Union

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WITH SIX FIGURES IN THE TEXT

Received 18 August 1960

SUMMARY

In this paper a new method is described for the examination of graft formation. The method depends in principle on the measurement of the tensile strength of the graft at intervals of 2 days. It has been applied to the analysis of the development of a simple graft, made by cutting across an internode of a tomato seedling and rejoining the separated portions. The method requires that the treatment be applied to a group of a hundred seedlings from which samples are taken at intervals of 2 days. With this general technique and with the system used it has been shown that the development of the graft continues steadily after the second day of the experiment, that graft formation is most rapid when the different tissues of the stem coincide in the stock and the scion, and that the tensile strength of the graft is reduced markedly when the turgidity of the tissues is depressed. In the last section of the paper the significance of these several observations is discussed.

INTRODUCTION

THE formation of a graft union involves the fusion of two initially independent units into a single coherent system. This remarkable and important process has attracted considerable attention and much information about it has been assembled, particularly with regard to the conditions necessary for the attainment of the final state, and with regard to the anatomical changes by which it is accompanied (in this connexion see Rogers and Beakbane, 1957). The formation of a graft union, however, is a dynamic process with quantitative characteristics that change with time. Graft formation may indeed be compared with the general process of growth. Both lead to the establishment of a final state and both involve elaborate anatomical changes. But with both the elucidation of the mechanisms involved must depend on the analysis of quantitative changes that occur during the development to the final state.

The data that are presented here represent the results of a preliminary investigation of the dynamic aspects of graft formation, and they are derived from an examination of the most general characteristic of the process. In the formation of a graft the union becomes progressively firmer with time, and the progress of the graft may therefore be analysed by measuring the change in the tensile strength of the union. In empirical terms this involves a measurement of the change in the breaking strain, which is determined at intervals after the two members are brought together. The series of values obtained provide a measure of the development of the graft in much the

[1] Mr. Roberts died on 8 April 1960.

same sense as serial weight-determination may provide an indication of the progress of growth.

A graft may of course be formed between units from different morphological systems and between units from plants of different taxonomic status. In this investigation, however, a considerably simpler situation has been chosen for analysis. It is the situation of a graft that is formed between the upper and lower parts of a seedling internode, after this has been divided by a transverse cut. When the two portions have been separated they are secured in position with the cut surfaces in contact, and the progress of the graft is subsequently determined by successive measurements of the breaking strain. The procedure requires, of course, the establishment of a population, all the members of which have been subjected to the same experimental treatment. Random samples are taken at intervals from the group, the breaking strain is determined for each member of the sample, and a mean value is calculated for the sampling occasion.

The change in the breaking strain has been measured with a number of experimental variables, whose effects have been measured to determine the scope and limitation of the general technique. The variables have not been chosen to analyse any particular phases of the grafting process and the results obtained with them are therefore necessarily fragmentary. Nevertheless, they do indicate certain important aspects and within their limitations they are of some significance. In an early stage of the investigation the effect of the angle of the cut across the internode was examined. The possibility was considered that the firmest and most rapid union might be obtained with a cut at an angle of 45° to the vertical axis. In fact it was found that a cut at an angle of 90° gave much the same results as one at 45°, and since the cut at right angles is easier to apply this has been adopted throughout the investigation. However, the right-angle cut introduces the possibility of variation in the coincidence of corresponding tissues when the two surfaces are brought in contact, and the effects have therefore been examined of turning the scion from the same seedling through 180° and of joining stocks to scions from different plants of the same group. At a later stage in the procedure, after the sample is taken, the seedlings must be removed from the pots in which they have been growing and each prepared for the determination of the breaking strain. The manipulations involved necessarily occupy some time during which the states of the two components of the system may change. In particular, turgidity may decrease. Accordingly, the effects of decreases in turgidity have been examined by allowing the seedlings to wilt, and in another experimental series by freezing them, after removal from the pots and before the breaking strain is measured.

MATERIALS AND METHODS

The observations have all been made with tomato seedlings (var. 'Ailsa Craig'). Seed is sown in soil in trays and when seedlings carry fully expanded cotyledons they are transferred singly to soil in pots and allowed to develop to the stage of having two fully expanded foliage leaves. At this stage the

seedlings are used experimentally, the graft being in each case made after cutting across the first internode above the cotyledons. The cut is made with a safety-razor blade and the two surfaces are immediately brought into contact and secured in position.

The experimental period is never less than 10 days and seldom more than 12. In the standard procedure, after the 2nd-day samples are taken from a group at intervals of 24 hours. No observations are made on the 1st day since at this time there is no measurable cohesion. The variation in the breaking strain for any single sampling occasion is large and it has been found necessary to use a standard sample of 10 seedlings. A single experimental group which is sampled over about 10 days therefore involves about 100 seedlings. When a comparison with a control series is involved the whole experiment may require 200 or more grafted seedlings. When the necessary operations have been completed on the experimental plants they are first placed in a frame in which the atmosphere is kept saturated. After the second day the plants are transferred to a normal greenhouse which is heated in winter.

In an investigation such as this, techniques used in horticultural practice for securing together the two parts of the graft cannot be adopted. These techniques are unwieldy, they cannot be standardized and they require far too much time. The device has finally been adopted of holding the two separate portions in position in a more or less rigid tube formed from a strip of pliable plastic material and a clip. A plastic strip about 1·5 in. long and about 0·75 in. wide is folded around the two stem segments. The free ends which project to one side are held in a paper-clip which has wide flat gripping surfaces. The edges of the gripping surfaces are brought as near to the stem and as far along the edges of the strip as possible. This operation leads to the formation of a more or less rigid tube which grips the two portions together. With this arrangement the whole process from cutting to securing the two surfaces in position may occupy less than 1 minute.

In principle the breaking strain is measured by gripping the graft on one side and attaching to the other a pan to which weights are added until a break occurs. A simple device is used in which a cord which is stretched over a pulley is attached on one side to one end of the graft and on the other to a weight pan. The pulley is carried by scaffolding on a base-board, to which a clip is attached immediately beneath the end of the cord which is secured to the grafted stem. The 'weights' used are strips of lead which weigh either 5 g. or 10 g.

In the standard procedure, after the seedling is removed from the pot, soil is shaken off the roots and the leaves are cut off. Either immediately or after treatment the seedling is placed in position for measuring the breaking strain, the stem on one side of the graft being gripped in the clip at one end of the cord, and the stem on the other side in the clip secured to the base board of the apparatus. Until the grafted plant is in position the plastic strip round the graft itself is not removed. This operation, however, is now completed and the addition of lead strips to the weight pan can begin. If 5-g.

weights are used these are added singly at intervals of 5 seconds, if 10-g. weights they are added at intervals of 10 seconds. The addition of weights continues until the break occurs and the pan consequently falls on to the base board beneath it.

Within the experimental period the break always occurs at the junction across which the graft union is being formed, and the period is limited by the stages in the process at which this occurs. After about 12 days a break may occur in the stem at some point remote from that of the graft. At this stage the grafting process is considered to be complete. It is significant that in each series of data given in the next section the recorded final values do not reach a constant level, and this is no doubt due to the fact that in its final state the graft has a greater tensile strength than normal tissues of the stem, and that therefore before the final state is reached, with the technique of analysis used, a break occurs in a region different from that of the graft. It is important, however, for the interpretation of the data given here to notice that during the experimental period in which the break is in the graft all the histological changes associated with graft formation occur. During this period a phase of intense meristematic activity is completed and vascular continuity is established across the union. At the time when breaks occur in a part of the system other than the graft direct vascular connexion between stock and scion has already been fully developed.

The values given in the next section are each the mean of ten observations, and they represent the mean weights required to effect the successive breaks in a series, and they are referred to therefore as the breaking weights.

RESULTS

The results of four separate experiments designed to show the change in the breaking strain with time are shown graphically in Fig. 1. These experiments involve the simple conditions characteristic of most of the control series of the investigation. The stock and scion are from the same seedling, the scion is restored without rotation, and the breaking weight is determined without any treatment of the graft after removal of the seedling from the pot.

It is evident that the results from the four experiments differ considerably, but it may be emphasized that the four groups of determinations were not made with the same environmental conditions. The experiments were conducted at different times of the year, with consequent variation in the temperature and light conditions, and the differences in the results are no doubt a reflection of this variation.

All four groups of results show one important feature. While no measurable cohesion develops within the first 2 days, thereafter the breaking weight increases steadily with time. It is of some interest that on the eleventh day the average breaking weight may be surprisingly large. In one series it is over 2 kg. The value of the final breaking weight in each series is certainly consistent with the view that by the eleventh day most of the development involved in the formation of the graft has been completed.

It is clear that the data are not sufficiently critical to provide unequivocal evidence of changes in the rate of development during the experimental period. At the same time they suggest that such changes do in fact occur, and at two stages. It is probable that in the early stage, up to the 4th or 5th day, the rate of development of tensile strength is comparatively slow and that thereafter it accelerates considerably. This is suggested by all the curves

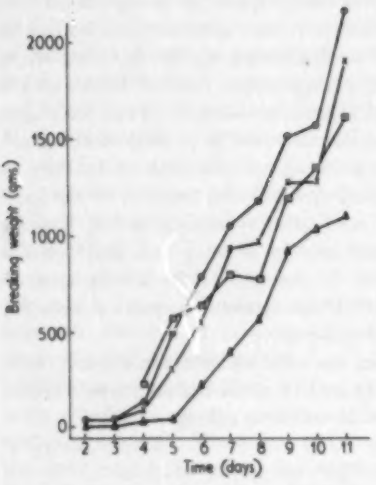

FIG. 1. Data showing change in breaking weight with time recorded in four separate experiments.

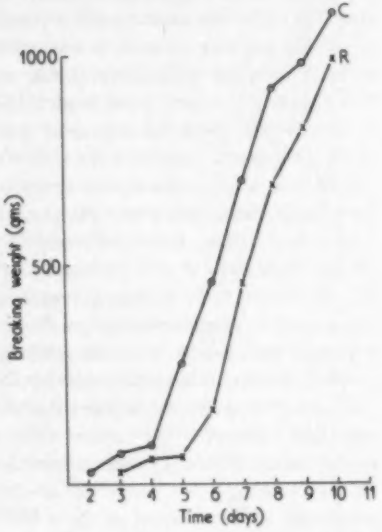

FIG. 2. Data showing effect of rotating the scion through 180° before joining it to the stock; *C*, control series; *R*, rotated series.

of Fig. 1 and by the control curves of Figs. 2, 4, 5, and 6. Clearly the evidence for an early lag phase is reasonably good. Certainly in no case is there any evidence for an initial rapid change with subsequent decrease in rate.

Secondly there is some evidence for a decrease in rate between the 7th and 8th days. This is suggested by three of the curves of Fig. 1 and by the control curves of Figs. 4, 5, and 6, the values of which were obtained with the same experimental conditions as those of Fig. 1. The conclusion, however, must be treated with some caution, since an apparent decrease in rate between the 7th and 8th days is not shown by one curve of Fig. 1 and by the control curve of Fig. 2. However, it is at least possible that a change in the situation does occur at about the 8th day. After this stage the rate of change in the breaking strain again becomes relatively rapid. If this conclusion is confirmed then it would indicate that development after the fourth day occurs in two stages, the first extending from the 4th to the 8th day, and the second from the 8th to the 11th day.

The results of one experiment designed to show the effect of turning the scion through 180° are shown graphically in Fig. 2. The data indicate that the treatment has a marked depressing effect on the development of the graft. The initial lag phase is prolonged until the 5th day and subsequently all values for the breaking strain are markedly lower than the corresponding values for the control.

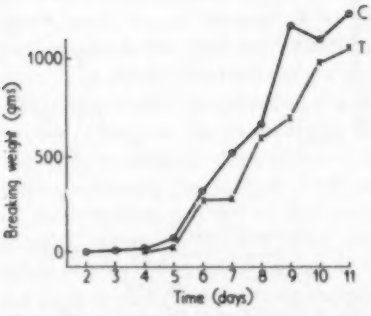

FIG. 3. Data showing effect of transferring scion of one seedling to stock of another; *C*, control series in which scion of same seedling has been rotated through 180°; *T*, transferred series.

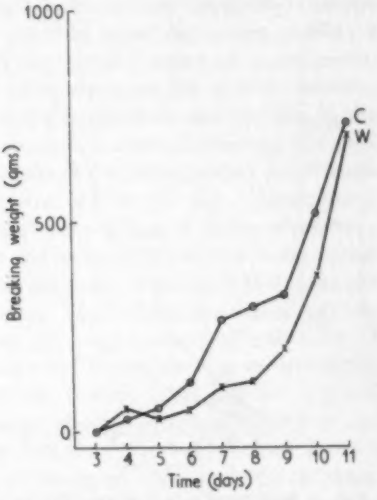

FIG. 4. Data showing effect of allowing seedling to wilt before determination of breaking; *C*, control series; *W*, wilted series.

The results of one experiment showing the effects of transferring the scion to a stock of another seedling are shown graphically in Fig. 3. The control in this case involves the condition of rotating the scion through 180° before placing it on the stock of the same seedling. It may be noted that the conditions of the control again prolong the initial lag phase to 5 days. A similar effect is observed with the series in which the scion is transferred. Throughout the experimental period the differences between the two sets of results are not large. Throughout, the values for the control series are the higher, and this being so it may be that graft formation is depressed by even slight differences in the genotypes of the stock and the scion. In the present instance this effect, if real, is only slight. On the other hand, since the effect of turning the scion is to depress the rate of graft formation it is probable that the values given by changing the scion are markedly lower than those that would be given by a standard control, and the results obtained therefore again emphasize the importance of coincidence of corresponding tissues for the most rapid development of the graft.

The results of one experiment designed to show the effect of wilting on the breaking strain are shown graphically in Fig. 4. The control series in this case involves the design of the experiments of Fig. 1. In the series in which wilting is applied the grafted seedlings, after removal from the pots, are allowed to remain on the laboratory bench until they are flaccid, and it is only when this state has been reached that the breaking weight is determined.

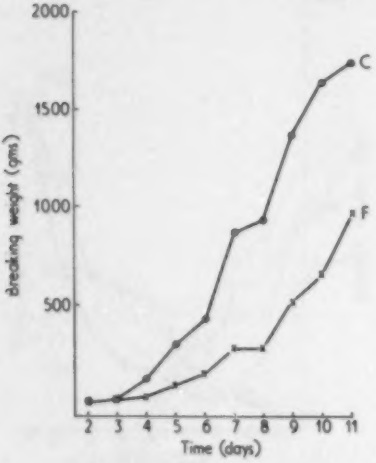

FIG. 5. First set of data showing effect of freezing the graft before determination of the breaking weight; *C*, control series; *F*, frozen series.

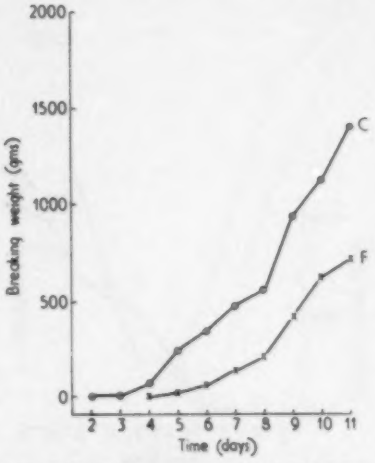

FIG. 6. Second set of data showing effect of freezing the graft before determination of the breaking weight; *C*, control series; *F*, frozen series.

The results show that this treatment tends to depress the tensile strength of the graft markedly.

The results of two experiments designed to show the effect of freezing on the breaking strain are shown graphically in Figs. 5 and 6. In both experiments the controls have the same design as the experiments of Fig. 1. In the frozen series, after removal from the pots and excision of the leaves, the seedlings are exposed for 2 hours to a temperature of less than 0° C. in the freezing compartment of a refrigerator. Subsequently the plants are brought back to room temperature before determination of the breaking weight. After thawing the seedlings are flaccid, and the tissues are impregnated with fluid. It is evident that freezing has a markedly depressing effect on the tensile strength of the graft. The effect in this case is considerably greater than it is with wilting. Wilting and freezing have both the same effect of reducing the turgidity of tissues and the reduction in the breaking weight may therefore in both connexions be attributed to this change. It is significant that the effect at least with freezing is relatively greatest in the early stages of the development of the graft. Thus in the experiment of Fig. 5, while the ratio

of breaking weight in the control and the treated material is 7·0 at 4 days, it is only 2·0 on the 11th day.

DISCUSSION

The data show that the formation of the graft union proceeds continuously and progressively from shortly after the two surfaces are brought into contact. At the same time the data also suggest (although not very decisively) that the process may involve at least two and possibly three distinctive phases: a first phase which, in the conditions of the present series of experiments, extends over the first 4 days, a second which occupies the interval between the 4th and the 8th days, and a third that extends from the 8th day to the end of the experimental period. In this connexion, some preliminary observations on the histological changes in the grafts of this investigation are of some significance. From the second to about the 6th or 7th day a longitudinal section across the graft shows a thick well-defined line of union which may stain more deeply than the tissues above and below it. This thick demarcation line is apparently composed of the crumpled walls of the cells that were cut by the passage of the razor across the stem. Until about the 6th or 7th day this deeply staining demarcation line is the only anatomical indication of coherence being established. Until about the 6th day there is no indication of meristematic activity in any part of the system. After the 6th day cell division begins in the tissues of the scion above the demarcation line and subsequently becomes apparent in the tissues of the stock below it. As a result of meristematic activity the plate of crumpled walls is disrupted, tissue continuity is established, and vascular elements differentiate in newly formed cells at the edges of the graft.

The data on change in breaking strain suggest that a change in the form of development occurs between the 7th and 8th days. Meristematic activity only begins on the 6th day, and it may therefore be suggested that the first two phases of development suggested by the breaking strain data refer to events in the junction line formed by crumpled cell walls. The enhanced rate in the development of tensile strength which characterizes the period from 8 to 11 days is evidently a consequence of meristematic activity and vascular development.

An elucidation of the conditions that determine attachment during the first two phases is clearly of considerable importance for the interpretation of the mechanism of graft formation. When the stem is cut the operation necessarily involves damaging all the cells in the track of the cutting instrument. These cells may release their contents which may then degenerate into a cementing medium; when the two surfaces are brought together the two sets of crumpled walls may be held together by this medium and thus form the demarcation line which is seen in longitudinal section. This interpretation is improbable for two reasons. First, the tensile strength of a union which depends on a mechanism such as this might not be expected to increase steadily over a period of 7 days and to be characterized by an initial

lag phase of at least 4 days. Secondly, with such a mechanism the coincidence of corresponding tissues should not be important. In fact it has been shown that even in the first phase, when meristematic activity is not involved, the rate of graft formation is depressed when corresponding tissues do not coincide. In this connexion the significant aspect of coincidence is probably that it brings into juxtaposition tissues with the same metabolic characteristics. This and the course of change in the breaking strain suggest that even during the first 6 or 7 days attachment is being determined by metabolic processes. It may be suggested that between the two sets of crumpled walls points of attachment are established which increase either in numbers or in area with metabolic control from the cells on either side. It is probable that the formation and growth of these attachments is at first slow but that this accelerates after the 4th day as a result no doubt of a change in the controlling tissues.

The active process of forming attachments between the crumpled walls clearly comes to an end about the 7th day, and it may be that the final phase can only begin when this process is complete. Certainly the increasing tensile strength of the last phase is a consequence of different cytological processes. In this phase the surfaces of freshly formed cells come into contact and adhere together. At the same time the coherence between stock and scion is further promoted by the development of vascular connexions.

It may be argued that the incidence of different mechanisms at different stages of the process is incompatible with the observed reduction in tensile strength with decrease in turgidity, since this might indicate that at all stages the tensile strength of the graft depends on contacts between turgid cells. The anatomical evidence shows clearly, however, that at least in the earlier stages contact between the external surfaces of cells from the two members of the union is not involved. The effect of a change in turgidity is undoubtedly due to non-uniform shrinkage in the stem as a whole. The transverse shrinkage in the two members to the union, particularly in the region of contact, is not likely to be uniform. Thus at the points where attachment has occurred, these are likely to be subjected to unequal shrinkages, or possibly to shrinkage in opposite directions on the two sides. In certain cases these stresses will promote breaks with a consequent decrease in tensile strength. In the terms of the interpretation proposed above, the strength of each attachment is likely to increase with time; hence, it may be suggested, the decreasing relative effect with time of decrease in turgidity.

REFERENCE

ROGERS, W. S., and BEAKBANE, A. B. (1957). Stock and Scion Relations. *Ann. Rev. Plant. Phys.* **8**, 217–36.

Growth and Cellular Differentiation in the Wheat Coleoptile

(*Triticum vulgare* L.)

I. ESTIMATION OF CELL NUMBER, CELL VOLUME, AND CERTAIN NITROGENOUS CONSTITUENTS

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WITH SIX FIGURES IN THE TEXT

Received 5 November 1960

SUMMARY

The numbers and volumes of cells were determined for consecutive stages in the growth and development of the wheat coleoptile (var. 'King II') when grown at 25° C. in darkness from soon after germination to senescence. Cell expansion occurred throughout growth and development up to 96 hours, and was accompanied by cell division between 18–60 hours. Evidence is presented that suggests there are two phases of cell expansion concerned in coleoptile growth.

Determinations were made at daily intervals from 24 to 120 hours after sowing of protein nitrogen, trichloroacetic acid soluble nitrogen (TCA-sol. N), ribonucleic acid (RNA), and desoxyribonucleic acid (DNA) in the coleoptile, and the results expressed on a per-coleoptile and per-cell basis. The maximum rate of net protein synthesis took place during or after the cell-multiplication phase of growth, depending on whether the results were expressed per coleoptile or per cell respectively. The ratio of protein nitrogen to TCA-sol. N changed considerably during growth, from 4·7 for young cells to 0·68 for mature cells.

The fluctuations in the values for RNA and protein are consistent with the template theory of protein synthesis and the DNA data are discussed in relation to polyploidy in differentiated cells. No significant difference was found in the nucleotide composition of RNA during the growth and development of the coleoptile.

INTRODUCTION

In a study of the biochemical changes which occur during the divergence of the two fundamental processes of growth and differentiation, work on plant cells has one important advantage over that on animals in that the results can be expressed easily on a per-cell basis. Moreover, in plants a comparison between the two processes is more practicable since they are usually separated in time and location. Biochemical growth studies on higher plants, in which the results have been expressed on a per-cell basis, have been pioneered by

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Brown and his co-workers, using pea and bean root and lupin leaf apex (e.g. Brown and Broadbent, 1951; Heyes and Brown, 1956).

In the present investigation the coleoptile was chosen for two reasons: first, because it is cellularly a relatively homogeneous organ and, secondly, because of its special significance in auxin bioassay. Avery, Piper, and Smith were among the first to realize the usefulness of the coleoptile as a biological material for which data could be expressed on a per-cell basis. In 1945 they estimated cell numbers in the oat coleoptile histologically, during its growth and cellular differentiation, and found that cell multiplication ceased (in the cortical cells) when the coleoptile was about 8 mm. high and thereafter growth was by cell elongation.

For this study the *wheat* coleoptile was selected. Fig. 1 shows a longitudinal section of a coleoptile as it appears 18 hours from the time of sowing at 25° C. and before cell division has begun. As in the oat coleoptile, the region in which cell division was subsequently seen to take place was a cylindrical zone confined mainly to the coleoptile tissues below the 'pore'. As long as division continued it proceeded with almost equal intensity throughout this region, with the exception of the outer epidermal cells, which were seen to enlarge but never to divide. Cellular differentiation during development of the coleoptile after germination is a simple process histologically, though not necessarily so biochemically or physiologically. During the growth of the seed, differentiation has already taken place into regions representing the outer epidermis, cortex, inner epidermis, and two vascular bundles; at germination the young embryo imbibes water and the coleoptile cells begin to expand. After a series of divisions, in certain regions, the cells continue to enlarge and differentiate until they are mature and then within 24 hours senescence sets in (i.e. when coleoptiles are grown in darkness as in these experiments).

EXPERIMENTAL METHODS

Coleoptile culture. Wheat grains of variety 'King II' were soaked in tap water for 2 hours prior to sowing in moist sand in zinc trays with loose-fitting lids. Undamaged grains of an average size, oriented so that their embryos were uppermost, were sown in rows about 1·5 cm. apart with 1·0 cm. between each seed. When sowing was completed the seeds were covered with a thin layer of sand and the sand compressed with a sheet of glass. The seeds were grown at 25° C.$\pm$0·5° C. and were left undisturbed until required.

Fresh weight. With the aid of a dissecting microscope for the early stages, and using the epiblast as a guide, the coleoptiles were excised from the young seedlings. The incision was made across the base of the epiblast at an angle of approximately 30° upwards from the horizontal plane (Fig. 1) and a complete, or almost complete, coleoptile was obtained. An additional guide was the fact that when the primary leaves were teased out (after first slitting the coleoptile lengthwise with a scalpel) if the incision had been too high they parted from the coleoptile tissue very easily, whereas if too low they could

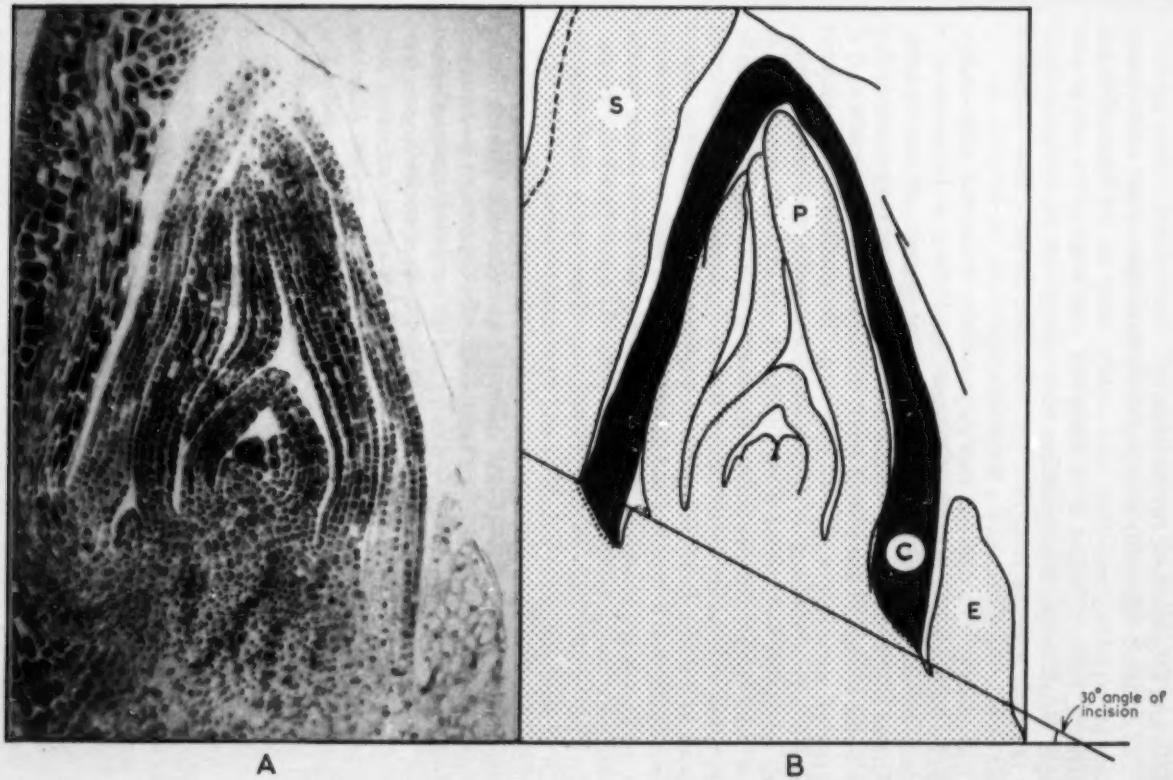

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FIG. 1. (A) Photomicrograph of a stained longitudinal section of the coleoptile and primary leaves 18 hours after sowing. (B) A tracing of (A) to illustrate where the incision was made to excise the coleoptile. S, scutellum; P, primary leaves; C, coleoptile, E, epiblast.

be removed only with difficulty. In practice the incision had to be made somewhere between these two extremes.

The dissected coleoptiles were placed on moist filter-paper until 10 were ready for weighing, then they were rapidly rolled on dry filter-paper and weighed. Three replicates of 10 coleoptiles were weighed for each growth stage. Fresh-weight determinations were carried out on coleoptiles at 6- or 12-hourly intervals, from 18 to 120 hours after sowing. (These periods of time include the preliminary 2 hours' soaking.)

Cell counting. Cell numbers were estimated after maceration of the coleoptiles by the method of Brown and Rickless (1949). Three replicate sets of 10 coleoptiles were macerated for each stage of growth and development. Chromic acid, as used by Brown and Rickless, was found to be unsatisfactory for wheat coleoptiles and instead 1 to 8 ml. (depending on coleoptile age) of 5 per cent. acetic acid in 2N. HCl were used. The addition of acetic acid was found to prevent shrinkage of the protoplasm and thereby facilitated counting. The coleoptiles were boiled for 2 minutes in the acid, with precautions to avoid volume loss, and then left standing overnight. Shaking was found to be insufficient to break up the coleoptiles completely, so the partially macerated tissues were drawn gently in and out through a series of hypodermic needles commencing with a large size and finishing with the smallest needle causing no appreciable cell breakage. The cell suspension was slowly drawn into the hypodermic syringe and a drop was run under the cover slip of a haemocytometer (as in blood-cell counting techniques). Five counts were made for each of three samples of a replicate and an average obtained.

For coleoptiles up to 48 hours after sowing an Improved Neubauer haemocytometer of 0·1 mm. depth was used and for older coleoptiles a Fuchs Rosenthal haemocytometer of 0·2 mm. depth. To ensure that no error was introduced, cell counts for coleoptiles 42 and 48 hours old were carried out with both types of haemocytometer. The two haemocytometers gave consistent results.

Cell volume. The average cell volume for a particular growth stage was estimated by dividing the fresh weight of the coleoptile by the corresponding cell number, on the assumption that the specific gravity of the cell was that of water.

Protein and TCA-soluble nitrogen estimations. The micro-Kjeldahl method of Ma and Zuazaga (1942) was employed. Total nitrogen was estimated directly by digesting whole coleoptiles and, from this value, trichloroacetic acid soluble nitrogen (TCA-sol. N) and nucleic acid nitrogen were subtracted to give protein nitrogen.

The TCA-sol. N value was obtained by grinding coleoptiles in 10 per cent. TCA, leaving the extract to stand at 2° C. for 2 hours, centrifuging, and determining the nitrogen in the supernatant. Nucleic acid N was calculated by first totalling the respective RNA and DNA phosphate values, determined as described below, and then multiplying the total by an arbitrary conversion factor of 1·68. This conversion factor was based on the average nucleotide

composition of the RNA fractions (Table III), the latter making up the bulk of the nucleic acid content. Any error due to the DNA having a different conversion factor would be small since the ratio of protein N to that of nucleic acid N was high.

Nucleic acid estimations. The preliminary extraction and separation of RNA and DNA was carried out by a modified Schmidt and Thannhauser (1945) procedure.

(*a*) *Removal of acid-soluble substances.* The coleoptiles were ground thoroughly with a little washed sand in 10 per cent. TCA (2 ml. per 0·6 g. fresh weight) in a small mortar and the material centrifuged at 12,000 *g* for 10 minutes. The process was repeated twice with fresh aliquots of TCA. The three supernatants containing the acid-soluble substances were discarded. A microscopic examination of the residue showed that from 98 to 100 per cent. of the cells were disrupted.

(*b*) *Removal of ethanol-soluble substances.* The residue was stirred with 95 per cent. ethanol (3 ml. per 0·6 g. fresh wt.) and centrifuged at 1,200 *g* for 10 minutes. The process was repeated and the supernatants discarded.

(*c*) *Removal of liquids.* The residue was boiled under reflux for 10 minutes with ethanol-ether (1:3, vol./vol.) (3 ml. per 0·6 g. fresh wt.) and then centrifuged at 1,200 *g* for 10 minutes. The process was repeated and the supernatants discarded.

(*d*) *RNA hydrolysis.* The residue was incubated at 37° C. for 18 hours with 0·3 N. KOH. This treatment hydrolysed the RNA to mononucleotides, while the DNA remained acid-precipitable. The KOH concentration is critical; with solutions stronger than 0·3 N., cytidylic acid is partially deaminated to uridylic acid (Davidson and Smellie, 1952), while if the concentration is much lower than 0·3 N. the RNA is incompletely hydrolysed. The pH of the residue was adjusted to 7 with 0·1 N. KOH before adding N. KOH and water to give a final concentration of 0·3 N. The volume chosen gave a concentration of approximately 70 μg. RNA-P per ml. This concentration was increased sixfold before application to filter-paper for electrophoresis (see below).

(*e*) *Separation of RNA mononucleotides from DNA.* The pH of the digest was adjusted to 1·0 with 60 per cent. perchloric acid and the extract left at 4° C. for 30 minutes before the precipitate containing DNA and $KClO_4$ was centrifuged down. The supernatant (RNA 1) contains the first batch of RNA mononucleotides. The precipitate was washed once with 0·1 N. HCl (using the same vol. as for RNA 1), and the mononucleotides in the supernatant (RNA 2) estimated separately from RNA 1. The volume of RNA 2 was corrected for the liquid remaining in the residue.

(*f*) *Estimation of RNA.* The two RNA extracts were adjusted to pH 4 with N. KOH and allowed to stand at 2° C. for 30 minutes. Insoluble $KClO_4$, which settled out, was centrifuged down. The pH of the supernatants was adjusted to 9 with saturated $Ba(OH)_2$ solution. Three volumes of ethanol were added and the solutions left standing overnight at 2° C., when the barium salts of the nucleotides separated and were centrifuged down. The barium

nucleotides were redissolved in water (about 1/6th of the original aqueous volume) and the solutions adjusted to pH 4 with N. H_2SO_4, liberating the free nucleotides and producing insoluble $BaSO_4$ (Parker, 1952; Thomas and Sherratt, 1956). The insoluble $BaSO_4$ was centrifuged down and the volume of the supernatant calculated by weighing.

Strips of Whatman No. 3 MM filter-paper (11 cm.×55 cm.) previously washed in buffer (see below) and dried, were used for electrophoresis. The RNA extract (0·06 ml.) was introduced along 2 cm. of the origin line on one half of the strip; the remaining width of the paper was used for blanks. The paper strip was immersed in buffer solution (formate buffer, 0·05 M., pH 3·8) almost up to the origin line and blotted between two sheets of filter-paper. The remainder of the strip was treated likewise and the two liquid fronts allowed to meet. Electrophoresis was carried out in an apparatus similar to that described by Markham and Smith (1952) using a current of 15 mA and a voltage source of 750V. d.c. for a period of 2 hours. The paper strips were allowed to dry at room temperature. Three replicate strips were made for each extract.

The nucleotide bands were located by photography on reflex document paper in ultraviolet light. The spots were marked out in pencil on the filter-paper and adjacent pieces of paper to each nucleotide of the same area were used as blanks. The nucleotides were eluted from the paper with 4 ml. 0·1 N. HCl at 37° C. for 18 hours. Extinctions of the eluate and the blank were read in the ultraviolet light and the quantity of each nucleotide (as phosphorus) calculated from the extinction coefficients listed below. These extinction coefficients apply to nucleotides dissolved in 0·01 N. HCl, but Rizvi (1957) found that 0·1 N. HCl gave the same extinction values.

Nucleotide	mμ	Extinction ε (P)	Ref.
Cytidylic	278	12,720	Ploeser and Loring (1949)
Adenylic	260	13,900	Volkin and Carter (1951)
Guanylic	260	11,800	,,
Uridylic	260	9,930	,,

(*g*) *Estimation of DNA*. The DNA was estimated using the diphenylamine colorimetric method of Dische (1930). The DNA precipitate, $KClO_4$, and the coleoptile residue, see above (*e*), were suspended in a measured volume of distilled water and twice the volume of the diphenylamine reagent added. The mixture was boiled for 10 minutes and allowed to cool. After centrifugation to remove coleoptile debris, the intensity of the blue colour in the supernatant was measured with a spectrophotometer.

Histology. The coleoptiles were fixed in formalin/acetic/alcohol and stored at 2° C. Subsequently they were dehydrated in an ethyl alcohol series and embedded in paraffin wax. Serial sections were made both transversely and longitudinally and stained with Delafield's haematoxylin.

RESULTS

Cell number. The cell complement per coleoptile increased from approximately 36,000 to 125,000 cells during growth and the increase followed a

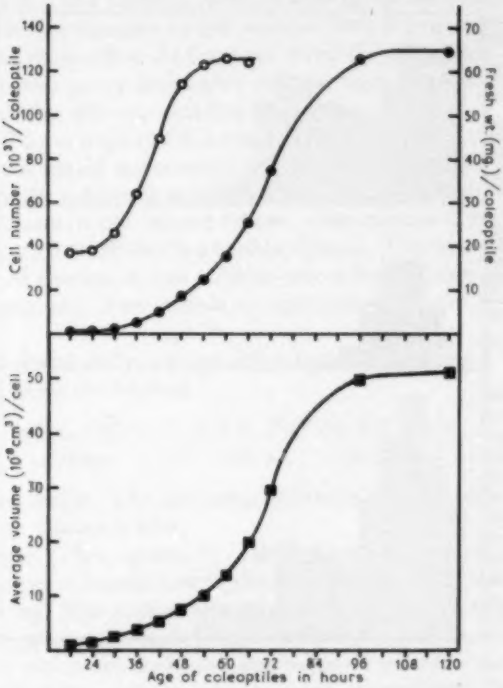

FIG. 2. Changes in cell number, coleoptile fresh weight, and average cell volume during the growth of wheat coleoptile. The 5% confidence limits are given in Table I.

(○ = cell number; ● = coleoptile fresh weight; ■ = cell volume)

TABLE I

Changes in fresh weight, cell number, and cell volume with age

(Values given ± twice S.E.)

Age of coleoptiles in hours	Fresh wt. per coleoptile (mg.)	Cell number per coleoptile × 10^3	Average cell volume cm.^{3} × 10^{-6}
18	0·357 ± 0·034	36·3 ± 0·52	0·98 ± 0·094
24	0·588 ± 0·046	37·6 ± 1·48	1·56 ± 0·136
30	1·12 ± 0·056	45·9 ± 5·16	2·44 ± 0·298
36	2·49 ± 0·026	64·0 ± 7·5	3·88 ± 0·466
42	4·95 ± 0·272	89·0 ± 2·0	5·57 ± 0·324
48	8·47 ± 0·152	114·0 ± 10·8	7·41 ± 0·800
54	12·25 ± 1·58	123·0 ± 7·6	10·04 ± 1·428
60	17·40 ± 1·41	126·0 ± 15·2	13·82 ± 1·90
66	25·1 ± 2·00	124·0 ± 7·02	19·85 ± 1·93
72	37·2 ± 2·32	..	29·54 ± 2·48
96	62·8 ± 3·64	..	49·70 ± 4·00
120	64·5 ± 1·72	..	51·2 ± 4·32

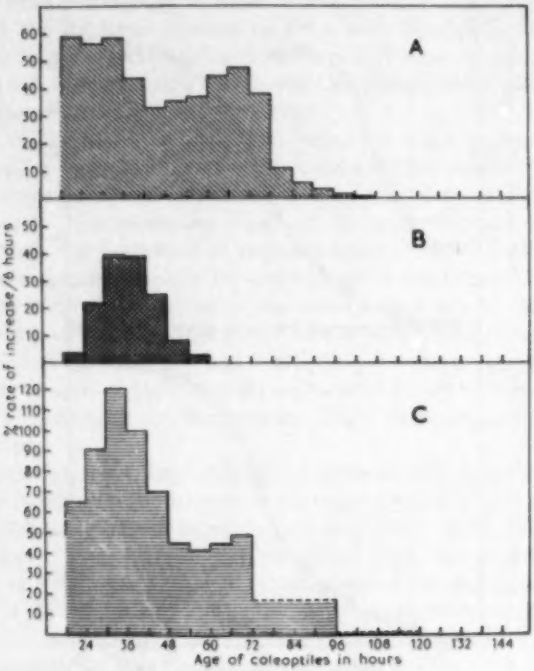

FIG. 3. Rates of change in (A) average cell volume, (B) cell number, and (C) coleoptile fresh weight during the growth of the wheat coleoptile.

TABLE II

Changes in N and P fractions with age

(Values given ± twice S.E.)

Age of coleoptiles in hours	24	48	72	96	120
PN per coleoptile ($g. \times 10^{-6}$)	0·661 ± 0·007	2·74 ± 0·294	5·02 ± 0·342	5·67 ± 0·538	5·64 ± 0·369
PN per cell ($g. \times 10^{-10}$)	1·801 ± 0·074	2·403 ± 0·34	4·048 ± 0·36	4·573 ± 0·50	4·548 ± 0·39
TCA-sol. N per coleoptile ($g \times 10^{-6}$)	0·143 ± 0·018	0·947 ± 0·129	4·24 ± 0·25	8·26 ± 0·31	8·50 ± 0·27
TCA-sol. N per cell ($g. \times 10^{-10}$)	0·38 ± 0·05	0·831 ± 0·137	3·42 ± 0·28	6·66 ± 0·449	6·85 ± 0·441
RNA-P per coleoptile ($g. \times 10^{-8}$)	33·11 ± 2·33	123·8 ± 3·92	163·4 ± 4·66	161·0 ± 5·00	81·4
RNA-P per cell ($g. \times 10^{-12}$)	8·81 ± 0·71	10·86 ± 1·08	13·18 ± 0·83	12·98 ± 0·83	6·54
DNA-P per coleoptile ($g. \times 10^{-8}$)	18·2 ± 1·53	67·6 ± 1·41	78·9 ± 6·66	69·0 ± 7·42	62·1 ± 2·6
DNA-P per cell ($g. \times 10^{-12}$)	4·84 ± 0·45	5·93 ± 0·57	6·36 ± 0·64	5·56 ± 0·67	5·01 ± 0·35

sigmoid curve (Fig. 2 and Table I). When coleoptile cell numbers were plotted as percentage rates of increase in cell number over 6-hour periods, the resulting histogram showed that the intensity of cell division rose to and reached a maximum between 30–42 hours after sowing, thereafter declining until at 60 hours none of the cells was dividing (Fig. 3*b*).

Cell volume. The average cell volume was found to increase about 52-fold during the growth period under study (i.e. from 18 hours after sowing until 120 hours, when the coleoptile is fully mature and has passed over to senescence). The increase in cell volume follows what appears to be a single sigmoid curve (Fig. 2), but in fact it is a double sigmoid. This was made apparent when the rates of increase in cell volume over 6-hourly time intervals were plotted as a histogram. Two periods of rapid cell expansion were indicated (Fig. 3*a*).

The standard errors of the average cell-volume means (Fig. 2 and Table I) were estimated using the formula

$$2\ \underset{\text{volume}}{\text{S.E. (\%)}} = \sqrt{\left(2\ \underset{\text{cell no.}}{\text{S.E. (\%)}}^2 + 2\ \underset{\text{fresh wt.}}{\text{S.E. (\%)}}^2\right)}$$

Coleoptile fresh weight. The data are presented in Figs. 2 and 3*c* and Table I and these will be discussed later.

Protein nitrogen (Figs. 4 and 5; Table II). The protein nitrogen increased 8·6-fold per coleoptile and 2·5-fold per average cell between 24 and 96 hours after sowing. The maximum rate of net protein synthesis occurred at different growth stages, depending upon whether the results were expressed on a per-cell or per-coleoptile basis. Per cell the maximum rate of increase took place during the cell-expansion period immediately following cell division (cf. Fig. 3*b* and *c* with Fig. 4) whereas per coleoptile the maximum rate of increase occurred during the cell-division period (i.e. 24–48 hours after sowing, cf. Fig. 3*b* and *c* with Fig. 5). The protein values indicated that net protein synthesis continued until 96 hours (when the coleoptile had reached 95 per cent. of its final length), but the increase between 72–96 hours was not significant at the 5 per cent. level in these experiments.

TCA-soluble nitrogen (Figs. 4 and 5; Table II). The TCA-sol. N increased 60-fold per coleoptile and 18-fold per cell between 24–120 hours after sowing. As with the protein N the periods of maximum rate of increase were from 24–48 hours after sowing per coleoptile and 48–72 hours per average cell.

RNA–P (Figs. 4 and 5; Table II). The RNA-P content per cell rose steadily from 24–72 hours (the increase over this period was highly significant; $p<0{\cdot}001$), levelled off, and then decreased rapidly. The percentage increases in RNA-P per cell for the two periods 24–48 hours and 48–72 hours after sowing were very similar (i.e. 23·3 per cent. and 21·4 per cent. respectively), whereas when the same data was expressed on a coleoptile basis the results indicated a more pronounced increase between 24–48 hours than between 48–72 hours (275 per cent. and 31 per cent. respectively). Unfortunately the wheat sample was exhausted before it was possible to replicate the RNA-P

content for coleoptiles grown for 120 hours and therefore the 5 per cent. confidence limits are not given for this stage in Table II.

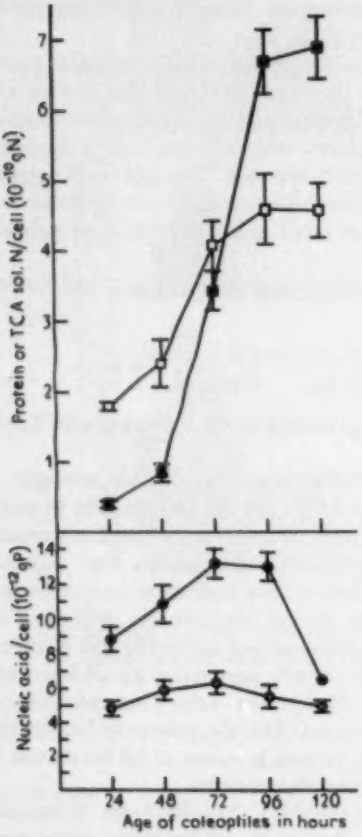

FIG. 4

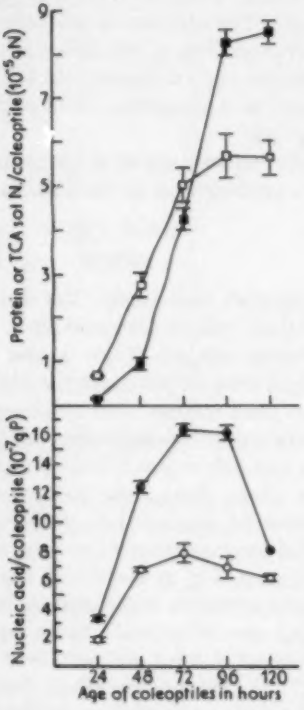

FIG. 5

FIG. 4. Changes in protein, TCA-sol. N, RNA, and DNA *per cell* during the growth of the wheat coleoptile (Table II). The vertical lines indicate the 5% confidence limits.
(□ = protein; ■ = TCA-sol. N; ● = RNA; ○ = DNA)

FIG. 5. Changes in protein, TCA-sol. N, RNA, and DNA *per coleoptile* during the growth of the wheat coleoptile (Table II). The vertical lines indicate the 5% confidence limits.
(□ = protein; ■ = TCA-sol. N; ● = RNA; ○ = DNA)

DNA-P (Figs. 4 and 5; Table II). Preliminary experiments established that in the later stages of coleoptile development (72-, 96-, and 120-hour extracts) there was a substance present in the DNA fraction which gave a green colour with the Dische reagent. It was possible to correct for this

contaminant since it was found to be present in the RNA fraction in a relatively pure state (see Fig. 6). The DNA estimate was corrected by reading the optical density at two wavelengths, 590 mμ and 710 mμ, and taking the difference to be proportional to the concentration of the DNA present. These two wavelengths were chosen since the optical density of the contaminant at 590 mμ equalled that at 710 mμ and by subtraction the error due to the contaminant was eliminated (Fig. 6). A standard curve was prepared relating

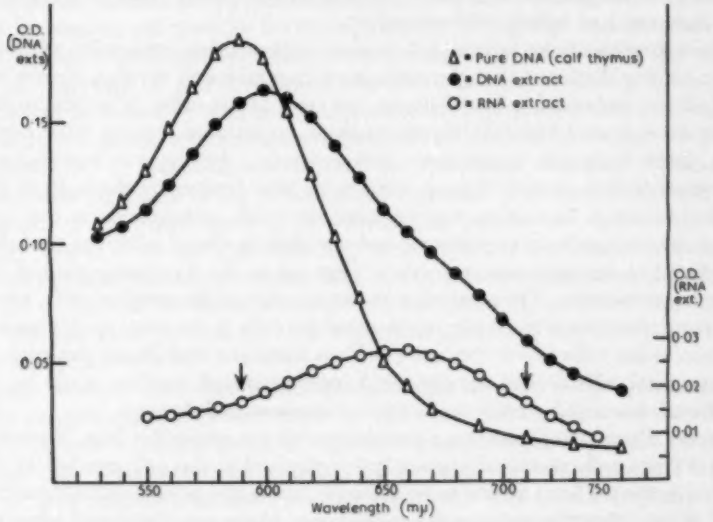

FIG. 6. Absorption spectra on calf thymus DNA, an unknown compound, and coleoptile DNA with the Dische reagent. The arrows indicate the two wavelengths at which the absorption due to the unknown compound is the same.

optical density (590–710 mμ) to micrograms of DNA-P with purified calf thymus DNA.[1]

The DNA-P content increased per cell and per coleoptile until 72 hours and then gradually declined. The increase per cell between 24–72 hours was significant ($p<0.01$) and so was the decline between 72 and 120 hours per coleoptile ($p<0.02$). The periods of maximum DNA-P synthesis (net) per coleoptile and per cell occurred during the period 24–48 hours after sowing and were 300 and 22 per cent. respectively.

DISCUSSION

It is important to realize that the coleoptile cells during embryonic growth have already passed through a prolonged period of cell division, so that in the fully grown grain the coleoptile comprises many thousand cells with the regions of the mature organ demarcated. After a short period of dormancy,

[1] Kindly supplied by Dr. A. S. Jones, Department of Chemistry, University of Birmingham.

growth recommences when the grain is placed under conditions favouring germination. As described in this paper, the cells of most of the coleoptile regions then undergo a period of meristematic growth lasting 42 hours (in var. 'King II'), during which the overall cell number per coleoptile increases from 36,000 to 125,000 cells. The absence of mitotic figures from the cells of the outer epidermis in stained sections of coleoptiles of different ages indicated that these cells do not divide, for they have probably completed this phase of development in the seed and at germination are ready to embark on the last stages of cellular differentiation.

Examination of the histological sections representing coleoptiles 18 hours after sowing disclosed that the main zone in which cell division occurs is a cylindrical one and implicated 80–90 per cent. of the cells. The cells in this zone were characteristically meristematic in appearance, having large nuclei and dense granular cytoplasm. Subsequently, during the meristematic phase of growth, mitotic figures were to be seen frequently throughout this zone. Although no mitotic figures were observed, certain cells in the apex were meristematic in appearance and division in these cells was probably restricted to compensatory growth in response to the increasing girth of the growing coleoptile. The remaining 10–20 per cent. of the original cells, which were never observed to divide, represented the cells of the outer epidermis and certain apical cells above the 'pore'. Thus assuming that 80–90 per cent. of the original cells divide, the threefold increase in cell number could be interpreted as resulting from two or three waves of cell division.

Avery, Piper, and Smith (1945), working with oat coleoptiles (var. 'Victory'), found from cell counts of histological sections that the cell number of the cortex increased from 25,000 to 75,000 cells during the growth and differentiation of the coleoptile and that the cell-division phase was completed when the coleoptiles were approximately 8 mm. long. In the studies described in this paper the cell-number estimate, as mentioned above, included all regions of the coleoptile and the cell-division phase was completed when the coleoptile was 60 hours old and approximately 17 mm. long. This discrepancy in the length of coleoptile at which cell multiplication ceases could be explained by either (i) an inherent difference in the growth patterns of wheat and oat coleoptiles, or (ii) that cell division in the cortical region is completed in the wheat coleoptile when 8 mm. long (42 hours after sowing) and that the increase in cell number between 42–60 hours is the result of cell division in the vascular bundles.

The average cell volume increased throughout the growth and differentiation of the coleoptile. From these data the rates of increase in cell volume for 6-hourly periods were calculated and the results depicted as a histogram (Fig. 3*a*). However, this histogram is misleading since it seems to suggest that there are two periods of rapid cell expansion with an intermediate period in which the growth-rate is slower. The decline in growth-rate between 36–60 hours is due to the incorporation in the method of estimation, of the negative growth-rate of cells in the process of division (i.e. the volume of a cell is approxi-

mately halved at division). A much better estimate of the average rate of increase in cell volume is given by the rate of increase in coleoptile fresh weight, ignoring cell number altogether. Reference to this histogram (Fig. 3*c*) shows that cell expansion can be divided into two growth periods: (i) when rate of cell expansion increases rapidly and then falls (18–48 hours after sowing), and (ii) a linear and slower period of cell expansion (48–72 hours) followed by a gradual decline until growth ceases (96 hours).

The initial increase in cell volume preceded and was out of phase with the rapid increase in cell number by about 6 hours (cf. Figs. 3*b* and *c*). Haber and Luippold (1960) in lettuce-seed germination studies have shown that the time of inception of cell division in respect to cell expansion in the root radicle can be varied by temperature treatments. They found that germination at 10° C. caused cell expansion to precede cell division by several weeks, whereas at 26° C. cell expansion and cell division almost coincided. Thus it appears that the length of the period of cell expansion prior to cell division is governed by the temperature of germination, at least in the root radicle of lettuce. Went (Went and Thimann, 1937) observed that, in cell tissue cultures, cell enlargement normally preceded cell division after the application of auxins and the results for the wheat coleoptile and lettuce roots, stated above, indicate that the same process (i.e. cell expansion preceding cell division) also occurs in intact organs.

Some workers have determined total nitrogen at different growth stages of the coleoptile and after correction for cell number have assumed that the values provide a reliable index of the amount of protoplasm present in the average cell. This assumption is only valid when the protein nitrogen/TCA-sol. N ratio remains constant throughout growth. The results in Table II show clearly that this is not so for the wheat coleoptile: the PN/TCA-sol. N ratio being 4·7 for young cells (24 hours after sowing) and decreasing to 0·68 in the fully enlarged cell.

Many workers have reported that protein synthesis continues during cell extension (i.e. cell extension following cell division) and the results reported here support this conclusion (Frey-Wyssling and Blank, 1940; Heyn, 1940; Brown, 1946; Thimann and Christiansen, 1950; Robertson and Turner, 1951; Brown and Broadbent, 1951; Heyes, 1960). The net protein synthesis per cell was found to be higher during cell extension following cell division (80 per cent. increase between 48–72 hours) than during the meristematic phase of growth (33 per cent. increase between 24–48 hours). When the results were expressed as net protein synthesis per coleoptile the reverse was found, the protein content increasing more rapidly during the meristematic phase (300 per cent.) than during the cell extension following cell division (85 per cent.) (Table II).

The RNA content per cell during growth was consistent with the theory that RNA forms a template for protein synthesis (Haurowitz, 1949). Thus the decline in RNA-P synthesis after 72 hours was reflected within 24 hours by a falling-off in protein synthesis (Fig. 4).

If one postulates that the proteins synthesized by dividing cells are different from those occurring during differentiation, one might expect to find a difference in the nucleotide composition of RNA. To investigate whether the nucleotide composition of RNA varies with the growth and differentiation of the cell, the values of each nucleotide per 100 moles were calculated for each stage of growth. As can be seen from Table III, very little difference was found, although as demonstrated by Heyes (1960) for pea root segments, there was a slight increase in the purine/pyrimidine ratio as the cell matures. The

TABLE III

Nucleotide composition of RNA

(Molar ratios calculated to a total of 100·0)

Age of coleoptiles in hours	24	48	72	96
Adenylic acid	23·40	24·10	24·50	23·70
Guanylic acid	29·90	29·20	30·20	31·05
Cytidylic acid	24·95	23·40	21·50	22·65
Uridylic acid	21·75	23·30	23·90	22·70
purine/pyrimidine ratio	1·14	1·14	1·20	1·21
6-amino/6-keto ratio	0·935	0·905	0·850	0·860

values only indicate an average nucleotide composition and do not rule out the possibility that RNA molecules of different nucleotide proportions are present in the cell (Heyes, 1960).

No attempt was made to elucidate the nucleotide composition of DNA during this study and only total DNA-P values were estimated for each growth stage. Holmes, Mee, Hornsey, and Gray (1955), who carried out an intensive study of the DNA-P content per cell in root segments of *Vicia faba*, concluded that the DNA content of fully differentiated cells was tetraploid. The DNA-P values depicted in Fig. 4 and given in Table II lend support to this hypothesis. As these workers have argued, the DNA content of meristematic tissue should, on average, be higher than non-dividing tissue, since the DNA in the nucleus has been found to double prior to mitosis. Since the results presented here show an increase in DNA-P content per cell during and following division, then it follows that the content of DNA-P per differentiated cell is greater than the diploid amount. The degree of polyploidy cannot be calculated since the time periods for the various stages of the mitotic cycle are not known and were not determined for the wheat coleoptile. The decrease in DNA content per cell after 72 hours was probably the first indication that senescence had commenced. This decrease was accompanied by a slight fall in RNA content and 24 hours later by a decrease in protein per cell. Thus there appears to be a sequential degradation of these three cell components during senescence: firstly DNA, secondly RNA, and lastly protein.

Finally, it is of interest to note that, despite the greater average cell volume, the DNA, RNA, and protein content of the mature coleoptile cell are of the same order of magnitude as found by other workers for mature cells in roots (Table IV).

TABLE IV

The average cell volume and the content of DNA, RNA, and protein in mature plant cells

	Average cell volume $cm.^3 \times 10^{-7}$	DNA $g. \times 10^{-11}$	RNA $g. \times 10^{-11}$	Protein $g. \times 10^{-11}$	Reference
Pea roots	2·1	5·0	18·70	147	Heyes (1960)
,, ,,	1·9	..	..	270†	Brown and Broadbent (1951)
Vicia faba roots	..	4·7*	18·0*	..	Holmes *et al.* (1955)
Wheat coleoptiles	5·1	6·4*	13·0*	320†	

* Assuming 10% P † Assuming 12·5% N

This study, the first of a series, was carried out primarily to form a suitable background to interpret experiments in which individual proteins and certain cell particles have been analysed using serological and analytical ultracentrifugal techniques. These results have been reported briefly elsewhere (Wright, 1960).

ACKNOWLEDGEMENTS

The author is very grateful for the encouragement and interest of Professor P. G. H. Gell throughout the course of this work, to Dr. G. F. Pegg for his constructive criticism of the manuscript, and to Miss A. H. Hudson for skilful technical assistance. The work was carried out under a grant from the Nuffield Foundation.

LITERATURE CITED

AVERY, G. S., PIPER, M., and SMITH, P. (1945). Cell number in successive segments of *Avena* coleoptiles of different ages; Material for the biochemist. *Amer. J. Bot.* **32**, 575.

BROWN, R. (1946). Studies on germination and seedling growth. III. Early growth in relation to certain aspects of nitrogen metabolism in the seedling of barley. *Ann. Bot.*, N.S. **10**, 73.

—— and BROADBENT, D. (1951). The development of cells in the growing zones of the root. *J. Exp. Bot.* **1**, 249.

—— and RICKLESS, P. (1949). A new method for the study of cell division and cell elongation with preliminary observations on the effect of temperature and nutrients. *Proc. Roy. Soc.* B. **136**, 110.

DAVIDSON, J. N., and SMELLIE, R. M. S. (1952). Phosphorus compounds in the cell. 2, The separation by ionophoresis on paper of the constituent nucleotides of ribonucleic acid. *Biochem. J.* **52**, 594.

DISCHE, Z. (1930). Colour reactions for thymonucleic acid and its determination in animal organs. *Mikrochemie*, **8**, 4.

FREY-WYSSLING, A., and BLANK, F. (1940). Growth of protoplasm and nitrogen migration in the coleoptile of *Zea mays*. *Nature*, **145**, 974.

HABER, A. H., and LUIPPOLD, H. J. (1960). Separation of mechanisms initiating cell division and cell expansion in lettuce seed germination. *Plant Phys.* **35**, 168.

HAUROWITZ, F. (1949). Biological problems and immunochemistry. *Quart. Rev. Biol.* **24**, 93.

HEYES, J. K. (1960). Nucleic acid changes during cell expansion in the root. *Proc. Roy. Soc.* B. **152**, 218.

—— and BROWN, R. (1956). Growth and cellular differentiation. In *The growth of leaves*. (Ed. F. L. Milthorpe.) Butterworths Scientific Publications, London.

HEYN, A. N. J. (1940). The physiology of cell elongation. *Bot. Rev.* **6**, 515.

HOLMES, B. E., MEE, L. K., HORNSEY, S., and GRAY, L. H. (1955). The nucleic acid content in the meristematic elongating and fully elongated segments of roots of *Vicia faba*. *Exp. Cell Res.* **8**, 101.

MA, T. S., and ZUAZAGA, G. (1942). Micro-kjeldahl determination of nitrogen. *Industri. Engng. Chem.* (*Anal.*), **14**, 280.

MARKHAM, R., and SMITH, J. D. (1952). The structure of ribonucleic acids. I, Cyclic nucleotides produced by ribonuclease and by alkaline hydrolysis. *Biochem. J.* **52,** 552.

PARKER, G. (1952). A study of the breakdown of ribonucleic acid in tobacco-leaf extracts. Ibid. **51,** 389.

PLOESER, J. McT., and LORING, H. S. (1940). The ultraviolet absorption spectra of the pyrimidine ribonucleosides and ribonucleotides. *J. Biol. Chem.* **178,** 431.

RIZVI, S. B. H. (1957). *The role of phosphorus-containing compounds in the gram-staining reaction of micro-organisms.* Ph.D. thesis, University of Birmingham.

ROBERTSON, R. N., and TURNER, J. F. (1951). The physiology of growth in apple fruits. II, Respiratory and other metabolic activities as functions of cell number and cell size in fruit development. *Aust. J. Sci. Res.* **4,** 92.

SCHMIDT, G., and THANNHAUSER, S. J. (1945). A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. *J. Biol. Chem.* **161,** 83.

THIMANN, K. V., and CHRISTIANSEN, G. S. (1950). The metabolism of stem tissue during growth and its inhibition. III, Nitrogen metabolism. *Arch. Biochem.* **28,** 117.

THOMAS, A. J., and SHERRATT, H. S. A. (1956). The isolation of nucleic acid fractions from plant leaves and their purine and pyrimidine composition. *Biochem. J.* **62,** 1.

VOLKIN, E., and CARTER, C. E. (1951). The preparation and properties of mammalian ribonucleic acids. *J. Amer. Chem. Soc.* **73,** 1516.

WENT, F. W., and THIMANN, K. V. (1937). *Phytohormones.* Macmillan (p. 224).

WRIGHT, S. T. C. (1960). Occurrence of an organ-specific antigen associated with the microsome fraction of plant cells and its possible significance in the process of cellular differentiation. *Nature,* **185,** 82.

Dormancy of Rice Seed

I. THE DISTRIBUTION OF DORMANCY PERIODS

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WITH SIX FIGURES IN THE TEXT

Received 20 September 1960

SUMMARY

The mean dormancy period is defined as the mean time taken from harvest for the individual seeds of a population to attain the ability to germinate under normal conditions. Evidence is presented which shows that the distribution of dormancy periods among the individual seeds of a pure line is normal. Since germination usually reaches 100 per cent., and because a normal distribution is symmetrical, a good estimate of the mean dormancy period can be obtained by observing the time taken from harvest to the point when 50 per cent. of the population is capable of germination as shown by a series of germination tests. The main error involved is due to the fact that there is no obvious way of deciding, to within a few days, when grain is ripe for harvest. Harvesting the seed prematurely tends to speed up the processes leading to loss of dormancy whereas late harvesting has little influence on the date on which the mean dormancy period is achieved; it follows that when estimating the mean dormancy period the error is much smaller if seed is harvested too early rather than too late.

Contrary to some previous reports, no correlation has been detected between 'duration' (period from sowing to modal flowering plus 30 days) and mean dormancy period among different varieties.

INTRODUCTION

MOST cereal seeds after harvest will not germinate under normal conditions until after a certain period has elapsed. In this condition the seed is usually described as dormant. Seed dormancy is an agriculturally useful character, as it prevents the grain from sprouting in the field if the weather is wet at the time of harvest. But it can be a disadvantage to rapid methods of plant breeding, when it is necessary to sow successive generations as soon as possible after harvest. The main reason for starting the present investigations was to study dormancy in rice with a view to developing a practical method for shortening the period of dormancy for use in breeding programmes.

The discussion by Koller (1955) has perhaps made it necessary to justify the term 'dormancy' as used in the present context. Koller argues that the word dormancy should only be applied to the embryo and not to the seed or fruit as a whole. He suggests that when this is done and it is also accepted that there is a time factor in germination '. . . it is possible to discard "dormancy" as a superfluous term . . .'. In this criticism, Koller has restricted the

meaning of the term dormancy in a way with which some workers would not agree. In the present investigations the term dormancy has been retained because it is believed that it is useful to describe—in a less restricted sense than Koller would allow—a particular condition: that is, the inability of the viable and fully morphologically-developed embryo to germinate under what would, at a later stage, be ideal conditions. The factors which prevent the embryo from germinating may be present within the embryo itself or may be located in other parts of the dispersal unit; but functionally the precise location of the factors inhibiting germination is irrelevant. The dispersal unit of the rice plant is a caryopsis surrounded by the attached lemma and palea. In the agricultural sense this is the 'seed', and from the practical point of view it is the ability of the embryo to germinate when contained within this complete unit which is the important consideration.

For convenience, the word 'seed' will be used in this paper in the agricultural sense described above and not in the restricted technical sense later imposed on the word by morphologists.

MATERIAL AND METHODS

The species *Oryza sativa* L. is usually considered to be divisible into at least two sub-species: tropical varieties are normally considered members of the *indica* sub-species and sub-tropical varieties members of the *japonica* sub-species. All the varieties used in the investigations described here, except one (which is noted in the text), are members of the *indica* group.

The description 'standard germination test' applies to the following technique: 100 seeds are distributed equally between four 9-cm. petri-dishes; the bottom of each petri-dish is lined with one 9-cm. circle of Whatman No. 1 filter paper and irrigated with 10 ml. of distilled water; the seeds are incubated for 7 days at 32°±1° C.; during incubation, two petri-dishes are kept in darkness and two are subjected to diffuse low-intensity daylight (maximum intensity about 300 lux). Where data from several successive germination tests are presented, the result of each test is plotted against the time when the test was started.

Moisture determinations were normally carried out by grinding seed in a coffee grinder and oven-drying at 105°±1° C. for 24 hours. In those cases where only an approximate estimate was required, moisture determinations were carried out by measuring the conductivity of the ground grain with a Marconi Moisture Meter. Moisture contents are expressed, as is usual in cereal technology, as a percentage of the original weight before oven-drying.

RESULTS

1. *The distribution of dormancy periods among individual seeds*

Various workers have adopted different criteria for measuring the dormancy period of a variety. One measure which has frequently been used is the time taken from harvest until 80 per cent. germination is achieved (Chandraratna

et al., 1952; Buenaventura, 1956). There has been a certain amount of confusion in the literature in discussions concerning the rate at which seeds break dormancy, and the implications of the shape of the curve when per cent. germination is plotted against time have not always been fully understood. In order to clear up misunderstanding, and also to be in a position to make best use of future results, it was decided that it would be profitable to investigate the distribution of dormancy periods among individual seeds.

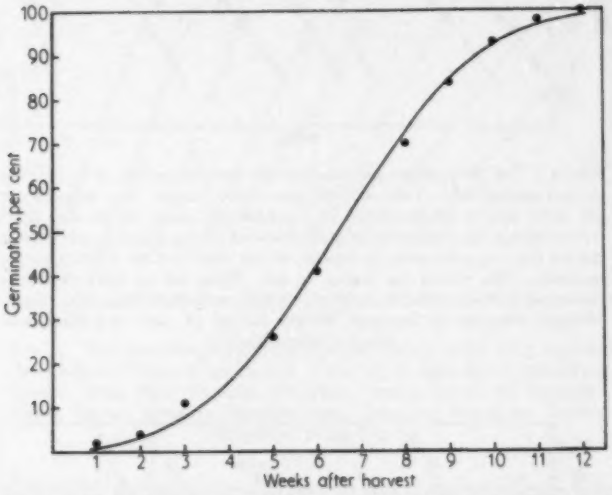

FIG. 1. The percentage germination of the variety 'Gabura' plotted against time. The curve was calculated from the experimental results (•) on the assumption that they represent a cumulative normal frequency distribution.

Investigations were made into a number of varieties and a typical example is illustrated in Fig. 1. This shows the results of germination tests carried out at intervals on sand trays in diffuse daylight at ambient temperature on seed which had been stored at ambient temperature (mean approximately 27° C.). The curve shown in this figure represents an estimate of the cumulative frequency distribution of the periods taken by individual seeds in the population to break dormancy; it is a theoretical curve calculated (Waugh, 1952) from the experimental results on the assumption that there is a normal distribution of dormancy periods among the individual seeds. It is evident that this curve provides a very good fit.

A large number of other rice varieties have been examined for the distribution of dormancy periods among the individual seeds, both by experiment and by examining the data published by other workers. When large numbers of results representing cumulative frequency distributions are being dealt with, a convenient method is to plot the results of the germination tests using

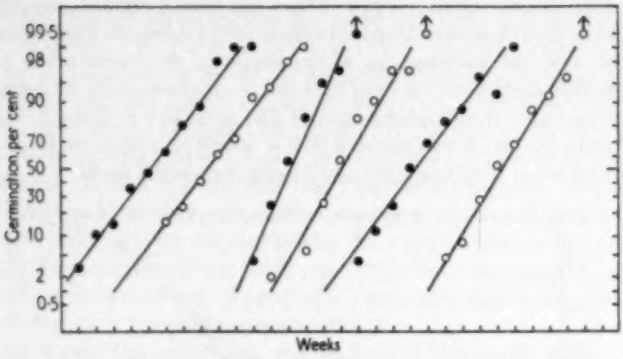

FIG. 2. The percentage germination (probability scale) of 6 varieties plotted against time. Data derived from Dore (1955). As a value of 100 per cent. cannot be illustrated on a probability scale, where this value was obtained experimentally it is represented on the graph as some value greater than 99·5 per cent. by a point at this value and an arrow pointing upwards. The curves are drawn by eye. From left to right the varieties are as follows: Rambutan, Radin Merah, and Anak Naga from South Malaya; Mayang Sa Gumpal, Seraup Kechil 48, and Seri Raja from North-Central Malaya.

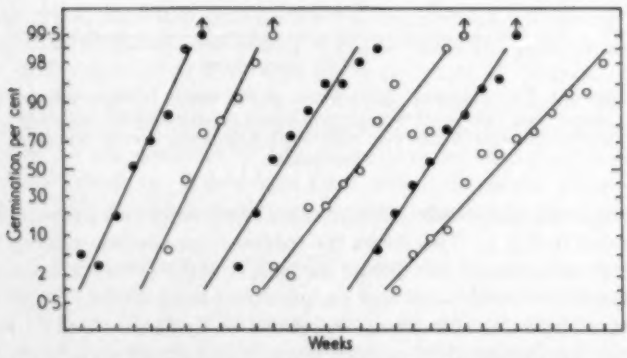

FIG. 3. The percentage germination (probability scale) of 6 varieties plotted against time. Data derived from Chandraratna *et al.* (1952). In three varieties the final level of germination reached was considerably below 100 per cent.; in these cases the experimental values have been corrected so as to ignore the non-viable proportion of the population. From left to right the varieties are as follows: Rathkarayan 3753, Vellai Illankalayan 2806 (corrected for final germination of 98 per cent.), and Molugusamba g–18, which are said to be relatively insensitive to photoperiod; Podiwi a–8 (corrected for 92 per cent. final germination), Kurulutuduwi b–18 (corrected for 98 per cent. final germination), and Mawi B–11, which are said to be highly sensitive to photoperiod. Further explanation as for Fig. 2.

a probability scale for percentage germination. When this is done, a normal distribution results in a straight line. Some examples of results replotted from the published data of other workers (Dore, 1955; Chandraratna *et al.*, 1952) are shown in Figs. 2 and 3. Fig. 4 shows some results which were obtained in this laboratory by storing and germinating seeds at ambient temperature; in this case the germination tests were carried out on sand trays

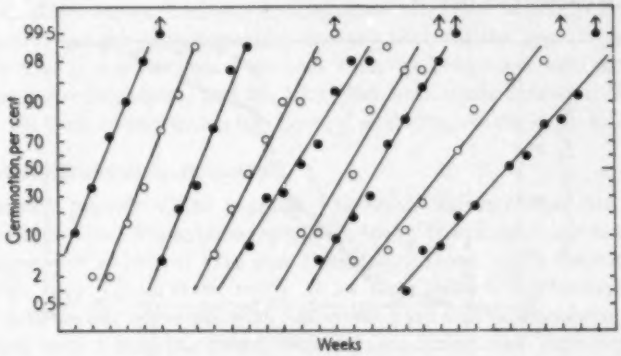

FIG. 4. The percentage germination (probability scale) of 9 varieties plotted against time. Original data. From left to right the varieties are as follows: Anak Ikan Gressing, Seri Raja, Seraup Kechil 36, Serendah, Morak Sepilai, Gantang, Samsep Leao, D99, and Bandakai. Further explanation as for Fig. 2.

in diffuse daylight. In general it will be seen that there is evidence for assuming that the distribution of dormancy periods within a pure line is normal. Very few exceptions to this generalization have been noted, and often in such cases there was reason to suspect that the sample of seed contained more than one genotype. As varieties differ in the length of their mean dormancy periods, one could expect a deviation from a normal distribution under these circumstances, unless there happened to be a large number of genotypes mixed at random.

From this discussion it follows that the gradient of the curve showing percentage germination plotted against time does not indicate the rate of breaking dormancy, as has sometimes been implied in the literature. A steep gradient shows that the spread or standard deviation of the distribution of dormancy periods among the seeds of a population is small, whereas a shallow gradient represents a wide spread of dormancy periods. If a single figure is to be used as a measure of the dormancy period of a variety, perhaps the most useful would be the 'mean dormancy period'. In the present investigations this is defined as the mean time taken from harvest for the seeds in a population to break dormancy. Since a normal distribution is symmetrical, and because a rice variety can usually be expected to attain approximately 100 per cent. germination, a good estimate of the mean dormancy period may be

obtained by measuring the time taken from harvest until the curve showing percentage germination attains a value of 50 per cent. The main error in this method of estimation is that the stage at which a variety should be harvested is a somewhat arbitrary and indeterminate point. Thus, in order to investigate some of the errors involved when seed is harvested at various stages of ripeness, an experiment was carried out to determine the influence of time of harvest on dormancy period.

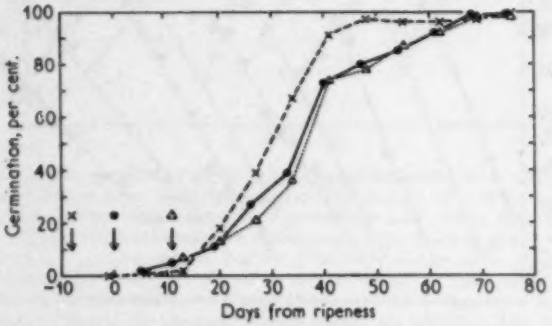

FIG. 5. The effect of harvesting date on dormancy. ↓ shows the date of harvest and the symbol used in plotting subsequent germination. Using normal agricultural criteria, the crop was considered to become ripe at day 0.

2. *The influence of time of harvest on dormancy*

A sample of grain was harvested from a plot of the variety Toma 112 on three occasions: (i) 8 days before it was considered to be ripe, (ii) at ripeness (as judged by ordinary agricultural criteria), and (iii) 11 days after ripeness. All samples were sun-dried after harvest to approximately 11 per cent. moisture content and then the seed was stored at ambient temperature in the laboratory. Standard germination tests were carried out at weekly intervals until maximum germination was obtained. The results of tests in the light and dark have been combined and are shown in Fig. 5. It will be seen that premature harvesting causes the seed to break dormancy on an earlier date than would occur if the seed is allowed to mature naturally on the plant: in this experiment bringing forward the harvesting date by 8 days brought forward the date on which 50 per cent. germination was achieved by 5 days (so that the mean dormancy period was 3 days longer). Delaying the harvesting date after the grain is ripe appears to have little effect on the date of breaking dormancy: the 11-day delay in harvesting only caused a delay of less than 2 days in the date on which 50 per cent. germination was achieved, and it is doubtful if this is significant. This experiment suggests then that once the grain is ripe the date of its detachment from the plant does not alter the date on which dormancy is broken; but harvesting before ripeness hastens the processes leading to loss of dormancy.

It is important to consider here the results of this experiment in relation to the general design of experiments on dormancy. Since the mean dormancy period is measured from harvest, late harvest would result in an apparent decrease in dormancy period, and the apparent decrease would be approximately equal to the delay. On the other hand, premature harvesting would result in an apparent increase in dormancy period; but in this case the increase would only be small as premature harvesting would be compensated to some extent by the resultant earlier breaking of dormancy. In other words, there is less error involved in the calculation of dormancy period if the grain is harvested too early than if it is harvested too late. This has been taken into account in the present investigations, and an effort has been made to ensure that any error in the time of harvesting has been, if anything, on the early side.

3. *Varietal differences in dormancy*

Dormancy periods differ markedly between different varieties (Shanmugasundarum and Venkatanarasing Rao, 1951; Shanmugasundarum, 1953; Chandraratna *et al.*, 1952; Oka and Tsai, 1955; Dore, 1955; Buenaventura, 1956; Lin, 1957; Umali *et al.*, 1957). It has been claimed that varieties which have a short duration from sowing to harvest have shorter dormancy periods than those with a long duration (Shanmugasundarum and Venkatanarasing Rao, 1951; Shanmugasundarum, 1953; Mudalier and Sundararaj, 1954; Umali *et al.*, 1957). Dore (1955) has disputed this relationship on the basis of measuring dormancy as the time taken to reach 98 per cent. germination; but nevertheless he states: 'Although not statistically significant, the mean maturation periods of varieties with different durations to 50 per cent. dormancy [i.e. the mean dormancy period as defined in the present paper] show the existence of a possible trend. The earlier maturing varieties tend to have a more rapid maturation of the seed than the slightly longer term varieties.' It has also been claimed that varieties of the sub-tropical *japonica* sub-species have shorter periods of dormancy than those belonging to the tropical *indica* sub-species (Tang and Chiang, 1955). Chandraratna *et al.* (1952) suggested that dormancy period is related to photoperiodic reaction—the relatively insensitive varieties having a short period of dormancy. This suggestion may be related to the previous two proposed correlations in the following way. *Japonica* or sub-tropical varieties are usually considered to be less sensitive to photoperiod than *indica* or tropical varieties (Beachell, 1943). There is also evidence that many varieties do not differ markedly in their minimum possible duration or in the optimum photoperiod necessary to obtain this minimum; it seems that differences in behaviour between varieties are due mainly to differences in photoperiod sensitivity (Chandraratna, 1954). As the optimum photoperiod appears to be about 10 to 10½ hours, this would mean that under most natural conditions in the tropics where day-length is longer than this, the types grown as short-duration varieties would be the least sensitive to photoperiod.

During the present investigations a survey has been made of the dormancy

of 137 varieties. All the varieties used were grown at Rokupr (9° 01′ N.) and were sown within 3 days of each other in June; they were transplanted, according to their development, after 5 to 7 weeks. One variety, Tai Chu 65 (which had the shortest mean dormancy period), was a *japonica* (or, as some systematists would prefer, a sub-*japonica*) variety; the remainder were *indica* varieties. The seed was harvested as soon as it was judged to be ripe. It was then sun-dried for 3 days and stored at ambient temperature in the laboratory.

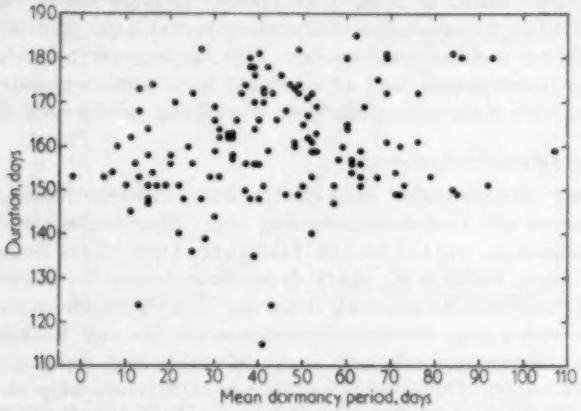

FIG. 6. The relationship between duration (period from sowing to modal flowering plus 30 days) and mean dormancy period for 137 varieties. One of the points on this diagram indicates that one variety had a mean dormancy period of less than 0; this was due to the fact that this variety showed more than 50 per cent. germination at harvest and the value assigned to it was obtained by extrapolation.

Germination tests were carried out at weekly intervals at ambient temperature in sand trays in diffuse daylight until the germination reached 100 per cent. or a value near to this. The mean dormancy period for each variety was estimated graphically; the values of percentage germination were plotted against time and the point was noted where the resultant curve crossed the line representing 50 per cent. germination. The duration—i.e. the period from sowing to ripeness—was calculated by a method which is common practice in rice breeding (Grist, 1959): the time from sowing to modal flowering was observed and 30 days was added to this value. This method is considered to be a more useful criterion of duration than direct measurement of the time from sowing to harvest because the period from flowering to ripeness can be altered considerably by weather conditions.

In Fig. 6 the mean dormancy period of each variety has been plotted against its duration. It will be seen that there is no apparent correlation between duration and mean dormancy period. The evidence suggests, therefore, that dormancy is not necessarily related to duration. Little information is available as to the photoperiodic behaviour of most of the varieties used—

except, as was discussed previously, that which can be implied from their durations. It is suggested that such evidence is not sufficiently reliable to warrant conclusions about the suggested relationship between photoperiod sensitivity and dormancy.

DISCUSSION

It has been shown that there is a marked variation in dormancy period between individual seeds which occurs even within a single sample of a pure line. Such random variation is perhaps made more understandable when it is remembered that all the flowers in a stand of genetically identical rice plants do not ripen at the same time: there is commonly about 5 days' variation in the time of flowering within the same panicle; furthermore, there is even greater variation between tillers and between plants. Consequently there is a considerable difference in age between individual seeds harvested on any one occasion. Although it is unlikely that this difference would account for all the variation in dormancy period encountered, it would explain at least a considerable proportion.

From the results of the investigations into the distribution of dormancy periods between individual seeds of a pure line it has been argued that if a single value is to be used to describe the dormancy behaviour of a variety there is much to be said in favour of using the mean dormancy period. However, it is realized that from the practical point of view the mean dormancy period may not be a sufficiently useful measure of dormancy on its own, because only 50 per cent. of the seeds will germinate at the end of this period. In breeding or agricultural work a higher percentage of germination than this is needed. In this connexion the present results suggest a method of estimating the time taken to reach a high percentage germination—say 98 per cent.—more accurately and easily than may be obtained by direct observation of the time taken for a small sample to achieve such a value. The method of direct observation is particularly inaccurate because of the nature of a normal distribution: the values which can be determined with least accuracy are those which approach 0 and 100 per cent. germination, as they depend on the behaviour of only a small proportion of seeds in the sample. A more accurate method is to calculate the parameters of the ideal curve from a few observations nearer the centre of the distribution and predict the desired level of germination from this. A convenient and rapid method based on this principle which has been used in this laboratory for predicting the time taken to reach a given high level of germination is as follows. The results of a series of germination tests are plotted against time, using a probability scale for percentage germination. A straight line is drawn through the points in the middle ranges of the distribution and the time of any given high level of germination may be predicted by extrapolation of this line.

The effect of the time of harvest on dormancy in rice seed has previously been investigated by Umali *et al.* (1957). These workers investigated a number of varieties by harvesting 4, 5, and 6 weeks after 'the time of heading'.

Measuring dormancy period as the time from harvest to maximum germination, they observed that '. . . the number of days or weeks by which harvesting was delayed corresponds exactly to the number of days or weeks by which the dormancy period is shortened . . .'. From this they concluded that the period between flowering and the time when harvested seeds will give maximum germination is fixed for a variety. The results recorded in the present paper agree with this conclusion, providing that harvesting is carried out when the seed is ripe or later. Under most conditions it is considered that ripeness is achieved about 30 days after modal flowering (Grist, 1959). But if the grain is harvested before this time, i.e. before maturity—a treatment not included in the experiments of Umali *et al.*—then a speeding up in the processes leading to loss of dormancy may be expected.

The results of the present investigation into varietal differences in dormancy periods and the relationship with duration (time from sowing to modal flowering plus 30 days) have suggested that no correlation exists between duration and dormancy period. This is an observation which does not agree with previous reports, particularly from India, where the lack of a dormancy period in short-duration varieties has been reported to be an agricultural problem (Ghose *et al.*, 1956). The present results therefore require some comment. Firstly, it should be pointed out that, in the experiment described here, varieties of extremely short duration which are common in India were not used; this is owing to the fact that it has not yet been found possible to cultivate successfully such varieties in West Africa. The variety with the shortest duration used in the experiment reported here had a duration of 115 days, whereas varieties with durations as short as 80 days are commonly used in India. It is possible then that our varieties lie outside the range of duration in which some correlation with dormancy period may be evident. Secondly, it should be mentioned that even where it has been reported that a short dormancy period, or lack of dormancy, is associated with short duration it is also evident that the association is not inevitable: Shanmugasundaram (1953), for instance, carried out some work on breeding short-term varieties resistant to sprouting and found that 22 out of 140 varieties of less than 120 days' duration were strongly dormant after harvest.

ACKNOWLEDGEMENTS

I wish to thank Mr. S. J. Wakka and Mr. G. S. Banya for technical assistance and the Director of Agriculture, Sierra Leone, for allowing publication of this work.

LITERATURE CITED

Beachell, H. M. (1943). Effect of photoperiod on rice varieties grown in the field. *J. Agric. Res.* **66**, 325.

Buenaventura, M. R. (1956). Dormancy periods of promising rice varieties. *Philippine Agriculturalist*, **39**, 558.

Chandraratna, M. F. (1954). Photoperiod response in rice (*Oryza sativa* L.). I. Effects on inflorescence initiation and emergence. *New Phytol.* **53**, 397.

CHANDRARATNA, M. F., FERNANDO, L. H., and WATTEGEDERA, C. (1952). Seed dormancy in rice. *Trop. Agriculturalist*, **108**, 261.

DORE, J. (1955). Dormancy and viability of padi seed. *Malayan Agric. J.* **38**, 163.

GHOSE, R. L. M., CHATGE, M. B., and SUBRAMANYAN, V. (1956). *Rice in India*, p. 112. Indian Counc. Agric. Res., New Delhi.

GRIST, D. H. (1959). *Rice*. 3rd edit., p. 101. Longmans, Green & Co. Ltd., London.

KOLLER, D. (1955). Regulation of germination in seeds. *Bull. Res. Counc. Israel*, **50**, 85.

LIN, C.-I. (1957). [Studies on the factors affecting germination in the panicle of rice.] *Mem. Coll. Agric. Taiwan Univ.* **4**, 13. *Plant Breed. Abstr.* (1959), **29**, abstr. 39?

MUDALIER, C. R., and SUNDARARAJ, D. D. (1954). Dormancy and germination of a few crop seeds. *Madras Agric. J.* **41**, 111.

OKA, H., and TSAI, K.-H. (1955). [Varietal differences in the dormancy and longevity of rice seedlings. Phylogenetic differentiation of cultivated rice. X.] *Japan. J. Breeding*, **5**, 90. *Plant Breed. Abstr.* (1956), **26**, abstr. 2403.

SHANMUGASUNDARAM, A. (1953). Studies on dormancy in short-term rices. *Madras Agric. J.* **40**, 477.

—— and VENKATANARASING RAO, M. B. (1951). Dormancy in rice seed and its importance in rice breeding. *Proc. First Scientific Workers Conf., Coimbatore*, p. 72.

TANG, W. T., and CHIANG, S. M. (1955). [Studies on the dormancy of rice seed.] *Mem. Coll. Agric. Nat. Taiwan Univ.* **4**, 1. *Field Crop Abstr.* (1958), **11**, abstr. 1509.

UMALI, D. L., MARQUEZ, L. D., and LANTICAN, R. M. (1957). Influence of degree of maturity on dormancy of the rice grain. *F.A.O. Int. Rice Comm., Paper Breed. 57:26*.

WAUGH, A. E. (1952). *Elements of statistical method*. 3rd edit., p. 177. McGraw-Hill, New York.

Growth Studies on Natural Hybrids between *Lolium perenne* and *Festuca pratensis*

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WITH THREE FIGURES IN THE TEXT

Received 18 August 1960

SUMMMARY

The growth-rates and chromosome numbers of plants taken from a natural population of hybrids between perennial rye grass and meadow fescue were determined. The growth-rates of diploid hybrids resembled that of rye grass while those of the triploids were similar to that of meadow fescue. Experiments on plants grown singly and in competition showed that fescue was adversely affected by low temperature and rye grass by low light and also by the reduced nutrient supply arising from competition.

The persistence of the sterile hybrids is explained by their recurrent formation coupled with greater longevity and vegetative spread, arising from growth characteristics intermediate between those of their parents. The persistence of rye grass and the disappearance of fescue when periodically grazed appear to result from the earlier growth and more rapid rate of tillering of the former. However, rye grass has fewer and smaller meristems available for regrowth if allowed to grow until anthesis, and under these conditions fescue dominates. The intermediate growth characters of the hybrids allow them to survive spring grazing more readily than meadow fescue and cutting for hay more readily than rye grass.

INTRODUCTION

NATURAL hybrids between *Lolium perenne* (perennial rye grass, $2n = 14$) and *Festuca pratensis* (meadow fescue, $2n = 14$) have been reported by several workers, notably Jenkin (1933, 1955) and Wit (1959). These hybrids are to be found along roadsides and in old, often damp, meadows undisturbed by cultivation. The hybrids may be either diploid or triploid but they are usually sterile despite normal chromosome pairing (Peto, 1933).

Artificial hybrids between the two species have been produced by Jenkin (1955), Crowder (1953), Wit (1959), and Reusch (1959); these, together with crosses involving other fescue parents of higher chromosome number, are of interest to plant breeders as a source of new varieties of agricultural importance. They also show the sterility found in the natural hybrids.

The present study was made to examine the vegetative growth of the hybrids and the manner in which their pattern of growth may influence their survival in a sward.

EXPERIMENTAL RESULTS

A population of natural hybrids in a riverside meadow at Thrussington, Leicestershire, was examined. The field, in which the hybrids are very

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frequent, floods in winter and is used for the production of hay or silage with grazing only during the autumn period.

A number of hybrid plants was taken at random from the field and clones established from them. These provided material for the experiments in which the main variable studied was the change in leaf number with time. The number of leaves on a plant may not, however, be an accurate guide to its dry weight and, since growth is more usefully defined in terms of dry-weight increments, the relationship between leaf number and final dry weight at harvest was considered in assessing the characteristics of a particular clone.

Eighteen of the hybrid plants were compared with single plants of S. 24 rye grass and S. 215 meadow fescue (Expt. 1). Individual tillers, each with four leaves, were planted singly in 7-inch pots filled with compost. The tillers were obtained by splitting clonal plants established earlier. The experiment was carried out in a greenhouse during the autumn of 1958 using a randomized block design with five replicates. The greenhouse was provided with electric heating which maintained the temperature only a few degrees above that prevailing outside and with illumination from fluorescent tubes giving an additional 0·56 cal./cm.^{2}/hr. at soil level for 16 hours each day. The conditions for growth, therefore, were far from good.

Chromosome numbers were determined from colchicine-treated root tips taken from each clone; fourteen of the hybrids were found to be triploid and four diploid. From fortnightly counts over a period of 10 weeks polynomials of leaf number (l) against time were fitted for each clone. These were of the form

$$l = a' + b'_1\xi'_1 + b'_2\xi'_2$$

where ξ'_1 and ξ'_2 are orthogonal polynomials in time and b'_1 and b'_2 provide independent estimates of slope and curvature respectively (Fisher and Yates, 1957). The main effects of time, clones and blocks, and the interactions of time with clones and clones with blocks were significant. Comparisons between individual clones (Table I) showed that rye grass had the highest relative growth-rate and those of the four diploids were higher than the majority of the triploids. The latter varied considerably and one (Hybrid 18) was particularly slow-growing under these conditions. Eleven weeks after planting all top growth was removed at one inch above soil level; a highly significant correlation ($r = +0{\cdot}86 \pm 0{\cdot}014$) was found between the leaf number and dry weight. In this experiment, therefore, the number of leaves present gave a reasonable estimate of growth.

Plants of each clone were then reduced to single tillers as before and the whole experiment was moved to another greenhouse without additional heat and light. Growth recommenced with the rising temperature of spring and counts of leaf number were made over a 16-week period (Table II). Rye grass again produced leaves the most rapidly and the diploids resembled it. On the other hand, meadow fescue was slower growing than all other clones including Hybrid 18. The growth behaviour of the clones relative to each other

TABLE I

Chromosome numbers and regression coefficients of $\log_e$ leaf number per plant on time (Expt. 1). (Regression calculated as $\log l = a' + b'_1 \xi'_1 + b'_2 \xi'_2$. Appropriate standard errors are given in brackets)

Clone	Chromosome number	a' ($\pm 0{\cdot}004$)	b'_1 ($\pm 0{\cdot}002$)	b'_2 ($\pm 0{\cdot}003$)
Rye grass (S. 24)	14	3·12	0·30	−0·04
Hybrid 1	14	2·81	0·28	−0·03
2	14	2·57	0·25	−0·01
5	21	2·47	0·22	−0·01
3	14	2·27	0·23	0·01
6	21	2·39	0·22	−0·01
7	21	2·44	0·22	−0·02
8	21	2·42	0·19	−0·02
4	14	2·38	0·21	−0·02
9	21	2·34	0·20	−0·01
Fescue (S. 215)	14	2·20	0·20	0·01
Hybrid 10	21	2·24	0·20	0·005
11	21	2·40	0·18	−0·02
12	21	2·07	0·19	0·03
13	21 + 1 fragment	2·21	0·18	0·004
14	21	2·11	0·16	−0·002
15	21	2·13	0·16	0·002
16	21	2·14	0·15	−0·02
17	21	2·16	0·13	−0·03
18	21	1·97	0·14	−0·04

TABLE II

Absolute growth-rates (leaves/week) with relative order of clones in brackets for Expt. 1. (10-week period in autumn and 16-week period in spring)

Clone	Autumn	Spring
R	8·24 (1)	8·37 (1)
1	6·04 (2)	6·34 (3)
2	3·84 (3)	7·72 (2)
5	3·08 (4)	4·53 (8)
3	2·86 (5)	5·79 (5)
6	2·82 (6)	4·49 (9)
7	2·68 (7)	4·48 (7)
4	2·40 (8)	3·96 (13)
9	2·26 (9)	4·23 (12)
11	2·20 (10)	3·55 (16)
8	2·20 (11)	3·37 (17)
F	2·10 (12)	1·38 (20)
13	2·04 (13)	4·24 (11)
12	2·00 (14)	6·22 (4)
10	1·88 (15)	3·76 (15)
15	1·68 (16)	4·47 (10)
14	1·44 (17)	3·94 (14)
16	1·36 (18)	5·03 (6)
17	1·16 (19)	3·16 (18)
18	0·74 (20)	3·01 (19)

appeared, therefore, to depend upon the levels of light and temperature operating during the period of growth. In Expt. 2, carried out during the late summer of 1959, four of the clones used earlier, S. 24, S. 215, Hybrids 1 (diploid) and 5 (triploid), were compared with five clones each of rye grass and fescue taken at random from the natural population. Uniform tillers were planted singly

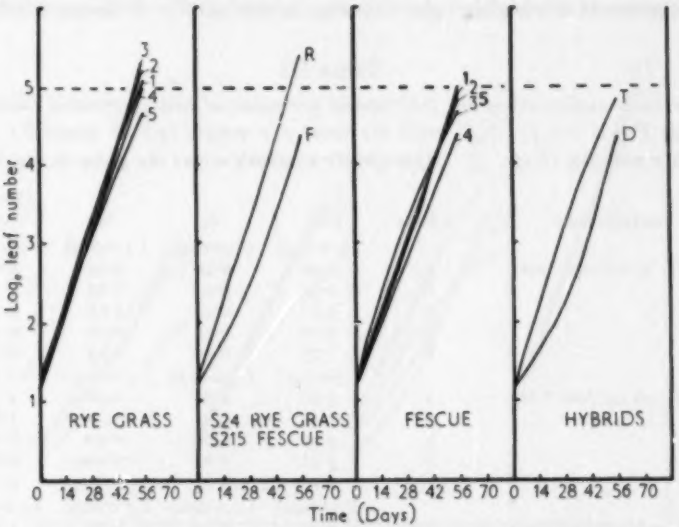

FIG. 1. Fitted curves for log$_e$ leaf number with time for plants of S. 24 rye grass (R) and S. 215 meadow fescue (F) compared with rye grass (clones 1–5), fescue (clones 1–5) and one diploid (Hybrid 1, D) and one triploid (Hybrid 5, T) from the same natural population.

in 7-inch pots filled with compost and the experiment was laid out as a randomized block with six replicates. Counts were made of leaf number at fortnightly intervals for 8 weeks. Significant effects of time, clones, blocks, and time with clones and blocks with clones were found. The fitted curves showed that the growth-rates within each group of the fescues, and within each group of the rye grasses, were similar (Fig. 1). The triploid and diploid hybrids were similar to the fescues but the diploid did not grow so rapidly as in Expt. 1. The general conclusions of Expt. 1 were supported by these results but it is noticeable that whereas a very marked difference existed in Expt. 1 between rye grass and fescue, the distinction was less obvious under the good growing conditions of this experiment.

The growth-rates under controlled conditions were compared in Expt. 3. Uniform single tillers were again chosen from the clones of S. 215 (F), S. 24 (R), the diploid Hybrid 2 and the triploid Hybrids 6 and 7, and grown at 15° and 25° C. with 2·5 and 6 cal./cm.^{2}/hr. visible radiation during a 16-hour day. There were four replicates of each clone in each environment. Leaf

number per plant was counted at fortnightly intervals for 12 weeks. Significant differences were found between clones under all four conditions of light and temperature and also between blocks under low light at both 15° and 25° C. Light influenced leaf production to a greater extent than temperature (Table III). Fescue had the lowest rate of leaf production under all conditions. But whereas rye grass showed its usual potential for rapid leaf production at the higher light intensity, it was similar to fescue under low

TABLE III

Regression coefficients of $\log_e$ leaf number per plant on time (regression calculated as $\log l = a' + b'_1\xi'_1 + b'_2\xi'_2$ with the mean dry weight (g.) of shoots 12 weeks after planting (Expt. 3). Appropriate standard errors are given in brackets

Environment	Clone	a'	b'_1	b'_2	Dry wt. shoots
		(±0·040)	(±0·029)	(±0·004)	(±0·170)
15° C. 39 cal./cm.²/day	1	2·26	0·46	0·05	0·62
	6	2·35	0·45	0·03	1·15
	7	2·21	0·42	0·05	0·79
	R	2·29	0·32	0·01	0·75
	F	1·73	0·29	0·03	0·57
		(±0·041)	(±0·016)	(±0·015)	(±0·250)
15° C. 96 cal./cm.²/day	1	3·56	0·82	−0·004	5·72
	6	3·43	0·72	−0·03	7·81
	7	3·29	0·68	−0·02	7·50
	R	3·22	0·72	−0·004	4·68
	F	2·57	0·57	0·02	4·24
		(±0·044)	(±0·040)	(±0·009)	(±0·470)
25° C. 39 cal./cm.²/day	1	2·69	0·54	0·01	1·33
	6	2·70	0·49	−0·08	3·14
	7	2·25	0·39	0·02	1·22
	R	2·18	0·38	0·07	0·71
	F	2·12	0·35	0·01	1·46
		(±0·049)	(±0·036)	(±0·023)	(±1·330)
25° C. 96 cal./cm.²/day	1	3·63	0·77	−0·02	7·43
	6	3·91	0·79	−0·08	13·32
	7	3·42	0·68	−0·03	10·10
	R	3·44	0·79	−0·001	7·44
	F	3·35	0·66	−0·05	8·77

light. The growth-rates of the diploid and rye grass were similar at high light intensity and that of the diploid was greater than rye grass under low light. Hybrid 6 had a faster growth-rate than the other triploid, Hybrid 7, under all conditions and was relatively less affected than rye grass by the variation in environments. In this experiment, however, the correlations between leaf number and dry weight of tops at harvest were not significant (r at 25° C., 96 cal./cm.²/day = +0·01±0·58; 25° C., 39 cal./cm.²/day = +0·52±0·49; 15° C., 96 cal./cm.²/day = +0·25±0·56; 15° C., 39 cal./cm.²/day = +0·50 ±0·49). On a dry-weight basis rye grass and Hybrid 1 were the lowest yielders under low light, irrespective of temperature. Fescue gave the lowest yield with the lower temperature irrespective of light intensity, indicating that

fescue has a higher temperature requirement than rye grass. The dry weights of the two triploids followed the same order as their leaf numbers. Hybrid 6, moreover, yielded the highest in all environments.

These results suggest that light and temperature are important factors determining the response of the two species and their hybrids. Furthermore, since the plants in a natural habitat are competing for nutrients and light, a study of the growth of individual plants does not yield good evidence of their

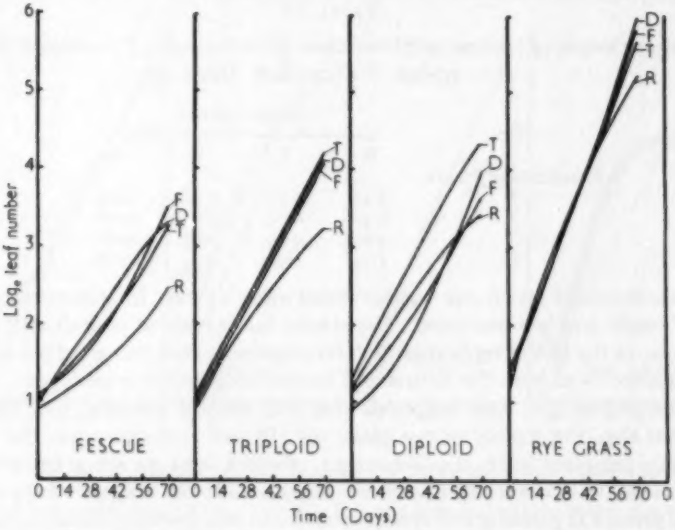

FIG. 2. Fitted curves of log$_e$ leaf number with time for central plants (Expt. 4). Central plants named at bottom of graph. Surrounding plants of diploid, triploid, rye grass, and fescue indicated by the letters D, T, R, and F respectively attached to the curves.

behaviour under sward conditions. Two attempts, of a similar nature, were made therefore to compare the growth of plants under competitive conditions. In Expt. 4 single tillers of the four clones—S. 215, S. 24, Hybrids 1 and 6—were surrounded by four tillers of each of the alternative types in separate 7-inch pots filled with compost. For example, one treatment consisted of a single central rye-grass tiller surrounded by four tillers of Hybrid 1. There were thus 16 treatments and 3 replications in a randomized block design. The experiment was conducted during the late summer of 1958 and during the following spring in the same greenhouse as that used for the first part of Expt. 1. The conditions for growth were therefore poor. Records of leaf numbers of the central plants made at fortnightly intervals showed that the main effects of time and clones were significant. The fitted curves (Fig. 2) showed that rye grass had the fastest relative growth-rate and, when grown as a surround, reduced the growth of all central plants much more than did the other clones. The diploid and triploid hybrids were intermediate between

the fescue and rye grass and there was little evidence that they differed in their competitive effect. The dry weights of tops at the end of the experiment confirmed the above results (Table IV).

In Expt. 5, which was carried out during the summer of 1959, lateral illumination was reduced by surrounding the pots with metal shields which were raised as the plants grew. The experiment differed only in the substitution of a commercial meadow fescue for S. 215 and the planting of six pots

TABLE IV

Mean dry weight (g.) of central plants where R = rye grass, F = meadow fescue, D = diploid, T = triploid. (Expt. 4)

	Central plants			
	R	F	T	D
Surrounding plants				
R	2·22	0·22	0·36	0·24
F	6·30	0·89	0·91	0·58
T	4·23	0·89	0·85	0·76
D	5·75	1·01	1·33	0·78

per treatment of which one was harvested every 17 days for determination of dry weight and leaf numbers. The results are subject to considerable error because of the lack of replication, but information on the growth of the species was gained from both the central and surrounding plants in each pot.

The dry-weight data suggested that the triploid material was initially heavier than the diploid or rye grass. At Harvest 1, for example, the mean weights per plant were: triploid, 0·04 g.; diploid, 0·03 g.; rye grass, 0·02 g.; fescue 0·05 g. It is likely that these differences in the weights of the tillers were present at planting and reflect variation in non-growing tissue, including reserve substances between the tillers which were selected only on the number of visible leaves. In this experiment, in which competition for both light and nutrient occurred, rye grass again had the fastest relative growth-rate over the initial stages (Fig. 3). Its growth-rate fell rapidly, however, almost irrespective of the type of surrounding plant. The solitary exception—when it was surrounded by the triploid—was probably an anomalous result since an examination of the growth of the rye-grass plants used as surrounds also showed the same reduction in growth. Evidently the rate of production of new leaf and stem tissue, reduced by increasing competition for light and mineral nutrients, no longer exceeded the rate of senescence of the leaves. This effect may well have developed first in rye grass since it formed leaves faster initially. It does not appear that the taller-growing fescue or triploid influenced rye grass through competition for light.

The diploid also showed the same decline in growth-rate, which was most marked with the triploid and fescue as surrounding plants. This suggests that these species may have exerted a shading effect as well as competing for nutrient. The difference between rye grass and the diploid may have arisen because the former grew faster and had already reached its maximum yield

before the fescue and triploid were big enough to cause shading. Thus the diploid shows the decline only from 56 days of growth whereas rye grass, at that time, had already reached its maximum growth.

Finally, growth of both the triploid and fescue was still continuing, irrespective of the nature of the surrounding plants, at the time the experiment ended, although it is noticeable that their rate of growth, when associated with rye grass, was falling rapidly. Since the leaf number per tiller was approximately the same for all four types considered, the mean leaf number per

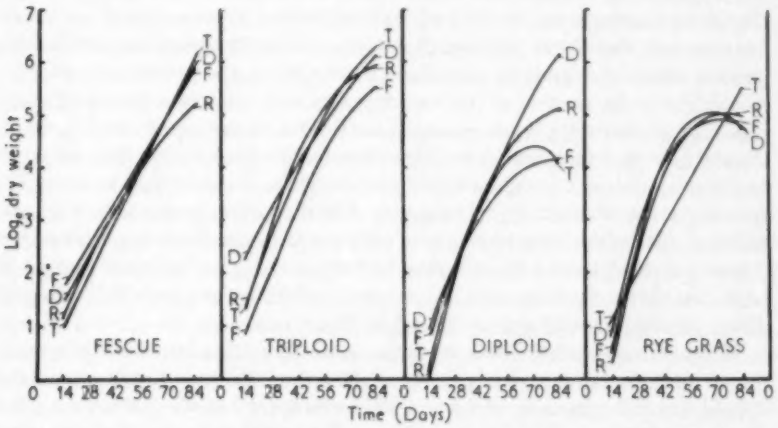

FIG. 3. Fitted curves of log$_e$ dry weight (*cg.*) with time for shoots of central plants (Expt. 5). Central plants are named at bottom of graph. Surrounding plants of diploid, triploid, rye grass and fescue are indicated by the letters D, T, R, and F respectively attached to the curves.

plant at 84 days (rye grass, 148; diploid, 110; triploid, 95; fescue, 47) indicated that the triploid and fescue make more growth per tiller than the rye grass and diploid. It is likely, therefore, that the increased size and delayed senescence of individual leaves may account for continued growth of the triploid and fescue. Fescue, which had the lowest rate of tillering of the four, maintained its initial growth for the longest time.

DISCUSSION

Although no estimates were made of the percentage seed set by hybridization, other work on artificial hybrids shows that the amount of seed formed and that germinating may vary widely with the particular plants chosen as parents. Values of 0–50 per cent. and 0–80 per cent. respectively were obtained by Reusch (1960) for seed set per number of flowers pollinated and for germination per number of seed formed. But since the frequency of illegitimate union would be much lower than that of legitimate union and as seedlings experience considerable difficulty in establishment it is likely that recurrent formation over a long period together with greater persistency of individuals

are the most important factors determining the frequency of hybrids in the sward. It is unlikely that the population is derived from a few hybridizations followed by rapid vegetative spread since several different hybrids were found in the small sample taken. Most of the hybrids are sterile and, although Jenkin (1955) has shown that introgressive hybridization can occur, the sterility of the diploid hybrids in this study and the regular chromosome number of the triploids suggests that they are not progeny derived from 'backcrosses'. The plants examined were of two types; the diploids with equal representation of rye grass and fescue chromosomes and the triploids with a ratio of 2:1 chromosome complements derived from either rye grass or fescue. It would be expected, therefore, that the diploid would be intermediate between its parents since the growth processes are largely under polygenic control. According to the content of the unreduced gamete the triploids should theoretically vary from types possessing growth-rates almost equal to rye grass to those whose growth-rates are similar to fescue. Those examined seemed to be of a festucoid type which, assuming that rye grass is most likely to act as the female parent (Reusch, 1959), suggests that the pollen grains would be unreduced. It would be interesting to examine triploids from a grazed rather than a hay field to test the likelihood of there being two distinct groups of triploids. (The chromosomes of rye grass and fescue are too similar to give direct cytological evidence of the origin of any triploid.)

There is considerable similarity between the hybridization of rye grass with fescue and that between two species of *Agrostis* (Bradshaw, 1958), where the hybrid was thought to combine the ability to spread of one parent with the density of tillering shown by the other and thus to have a greater adaptation to grazing conditions than its parents. Although the growth studies made on the *Agrostis* hybrids were not elaborate they showed the hybrid to possess the characters of both parents with perhaps an indication of heterosis. The experiments described in this paper show no evidence of heterosis for either leaf number or dry weight except where light conditions were limiting as in Expts. 3 and 5. It is, in fact, rare for a hybrid to possess a faster growth-rate than the most rapidly growing parent (Lewis, 1955). Furthermore, since the growth-rates of the hybrids studied were in general intermediate between the parents, it is impossible to ascribe, as in *Agrostis*, their success to having both of two characters possessed singly by their parents.

Unfortunately there is little evidence concerning the factors involved in the competition of species growing in an established sward. However, the likely sequence of events that occurs in the field during one growing season may be as follows. In spring the rye grass and diploid hybrids commence growing earlier and grow at a more rapid rate than the fescue or triploids. They will compete more effectively for nutrients, especially nitrogen, thus hindering the development of the slower-growing species (Donald, 1958). This process would lead, as it does in a heavily grazed sward, to the elimination of fescue and presumably also of the festucoid triploid, were it to continue from season to season. Compared with rye grass, species such as meadow fescue, Yorkshire

fog, tall oat, or the triploid hybrid are advantageously influenced by hay conditions; the difference between grazing and hay conditions must be due to some factor associated with flowering and the rate of regrowth following cutting. It is possibly reflected by the number and vigour of the buds available to continue growth after cutting.

Flowering occurs first in rye grass. It was shown in timothy grass that after flowering there was no further gain in dry weight and a net loss of leaf area occurred despite the formation of new vegetative tillers (Langer, 1957), while in a barley-persicaria association the unsuccessful competitor (persicaria) was able to develop more profusely following flowering of the aggressor (Aspinall and Milthorpe, 1959). Thus the effects of flowering at different times may contribute to the maintenance of a varied flora composed of species with different growth-rates.

Competition for light and for nutrients are clearly interacting systems (Aspinall, 1960) and both operate during the development of the sward. The parts of the plants most affected will be the latest-formed tillers arising from the axillary buds of main tillers. These shaded tillers are at first dependent on the parent plant for their mineral supply and photosynthate. It is likely that many will not develop while others make weak etiolated growth. This situation is far more likely to apply to rye grass than to fescue or the triploid as there are more tillers and each tiller makes less growth than with fescue. The propensity for tillering in rye grass and the diploid will be a disadvantage since new tillers develop under unfavourable conditions.

Eventually the sward is cut for hay and growth begins again from the basal buds which escape destruction. Regeneration of rye grass when cut at or after flowering is particularly slow (Davies, 1960; Evans, 1960). Although the explanation for this response is not known it may arise from the lack of carbohydrate and mineral nutrient reserves, a raised position of the buds due to etiolation, or simply to the buds of the recently produced tillers being less well developed. As the fescue and triploid produce fewer tillers and more of their buds are present on older, non-shaded tillers their development would subsequently be favoured. This may give fescue and the triploid sufficient advantage in colonizing the bare ground available after harvest to enable them to survive, despite the fact that their growth-rates per tiller during the late autumn and winter are lower than those of rye grass and the diploid.

The conclusions suggest that the survival of these hybrids is not due to any excessive growth covered by the term 'heterosis'. Instead the hybrids have growth habits intermediate between those of their parents. The possession of growth-rates intermediate between their parents enable them to survive, partially on account of the effects associated with flowering, which encourage suppressed species to grow, and also because their intermediate characteristics enable them to survive rather better than their more-specialized parents in the cycle of events which influence the number of meristems of each species present.

Further aspects of this problem require investigation. These include the

effects of various light and temperature conditions on the individual species, a detailed study of the mechanisms leading to the difference in rates of branching between the parent species, and the factors influencing regeneration after defoliation. An extension of the study of the development of individual plants, tiller by tiller, in a population containing hybrids, their parents and other species would give valuable information about the consequences of growth in mixed populations which is, at the moment, lacking.

ACKNOWLEDGEMENTS

We wish to thank Professor F. L. Milthorpe for his advice and helpful criticism during the course of this work and Dr. E. J. M. Kirby for reporting the presence of the hybrids examined. We are also grateful to Miss E. Lord and Mr. L. Heathcote for their considerable assistance.

LITERATURE CITED

ASPINALL, D. (1960). An analysis of competition between barley and white persicaria. II. Factors determining the course of competition. *Ann. Appl. Biol.* **48**, 637.

—— and MILTHORPE, F. L. (1959). An analysis of competition between barley and white persicaria. I. The effects on growth. Ibid. **47**, 156.

BRADSHAW, A. D. (1958). Natural hybridization of *Agrostis tenuis* Sibth. and *A. stolonifera* L. *New Phytol.* **57**, 66.

CROWDER, L. V. (1953). Interspecific and intergeneric hybrids of *Festuca* and *Lolium*. *J. Hered.* **44**, 195.

DAVIES, I. (1960). Growth stage and related factors in grassland management. *Rep. Welsh Pl. Breed. Sta.* 1959, 104.

DONALD, C. M. (1958). The interaction of competition for light and for nutrients. *Aust. J. Agric. Res.* **9**, 421.

EVANS, G. (1960). Density of grasses for seed production. *Rep. Welsh Pl. Breed. Sta.* 1959, 88.

FISHER, R. A., and YATES, F. (1957). *Statistical tables for biological, agricultural and medical research*. 5th ed. Oliver and Boyd, Edinburgh.

JENKIN, T. J. (1933). Interspecific and intergeneric hybrids in herbage grasses. Initial crosses. *J. Genet.* **28**, 205.

—— (1955) Interspecific and intergeneric hybrids in herbage grasses. XVI. *Lolium perenne* and *Festuca pratensis* with references to *Festuca loliacea*. Ibid. **53**, 379.

LANGER, R. H. M. (1957). Growth and nutrition of timothy (*Phleum pratense*). II. Growth of the plant in relation to tiller development. *Ann. Appl. Biol.* **45**, 528.

LEWIS, D. (1955). Gene interaction, environment and hybrid vigour. *Proc. Roy. Soc.* B, **144**, 178.

PETO, F. H. (1933). The cytology of certain intergeneric hybrids between *Festuca* and *Lolium*. *J. Genet.* **28**, 113.

REUSCH, J. D. H. (1959). Embryological studies on seed-development in reciprocal crosses between *Lolium perenne* and *Festuca pratensis*. *S. Afric. J. agric. Sci.* **2**, 429.

—— (1960). The effects of gamma-radiation on crosses between *Lolium perenne* and *Festuca pratensis*. *Heredity*, **14**, 51.

WIT, F. (1959). Hybrids of rye grass and meadow fescue and their value for grass breeding. *Euphytica*, **8**, 1.

Histological Studies on the Georeaction shown in the Xylem of the Main Axis of *Ricinus communis* L.

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Received 18 August 1959

SUMMARY

When the axis of castor-bean which has been fixed in a horizontal position for some time is rotated around its longitudinal axis and is then fixed in a new horizontal position, the zone of mucilaginous layers which was being formed (in a symmetrical crescent shape with its thickest portion situated at the topmost part of the xylem on the upper side), ceases further formation, and a new, less symmetrical, zone begins to form in the area which extends from the new upper side to the new lower one in the direction opposite to that of rotation. The greater the angle of rotation, the further the area where the new zone of mucilaginous layers is laid down extends; in the case where the horizontal axis is turned upside-down, the new zone of mucilaginous layers is formed symmetrically in the xylem on the new upper side. Moreover, if the axis once horizontally fixed is turned to a vertical position, the formation of mucilaginous layers stops on the old upper side and starts on the old lower one. However, formation on the lower side is soon discontinued.

As a possible physiological factor for producing these results, the effect of the accommodation of protoplasm to the preceding horizontal fixation upon the formative and metabolic responses to the stimulation of gravitational forces is discussed.

INTRODUCTION

It has been reported that axes of some herbs kept horizontally show an eccentric growth, presenting various histological differences between the upper half and the lower (Sato, 1956). More specifically, if the axis of castor-bean (*Ricinus communis* L.) is so treated, a reaction wood is formed on the upper side which bears well-marked characteristics—such as a formation of mucilaginous layers and a considerable decrease in thickness and lignification of cell walls—which are not found on the lower side at all (Sato, in press).

If it is true that these changes arise as a result of a direct effect of gravity, changes of growth in thickness and of tissue development will again be brought about, in proportion to the angle of rotation, when a horizontal axis is rotated around its longitudinal axis or is raised up from its original position. The present work was undertaken to investigate this possibility.

MATERIAL AND METHODS

Castor-bean seedlings were transplanted in rectangular wooden boxes, as described in an earlier report (Sato, 1956). When they had grown to a height

of 20 cm. or so, a prop was put up along each of their axes to prevent geotropic bending. These axes were then fixed in a horizontal position by turning the box sideways. Several days later, the plants were rotated through given angles by rotating the box, and were kept in the new position for the same period.

In each experiment 15–20 seedlings were used. The transections were taken from the portion near the base of the hypocotyl, where the growth in length had already finished and the growth in thickness was going on at the commencement of the experiment. For the histological observations, the combination of safranin and Delafield's haematoxylin was mainly used. The reactions with phloroglucin and HCl, and with zinc chloriodide, were also observed.

RESULTS

A. *Horizontal axis rotated around its own longitudinal axis by 45°, 90°, or 135°*

In every case, the zone of mucilaginous layers (Figs. 1, 2, and 3, m_1) which were formed in a symmetrical crescent shape with its thickest portion situated at the topmost part of xylem on the old upper side before the rotation (Figs. 1, 2, and 3, U_1), ceases further formation, and another zone (Figs. 1, 2, and 3, m_2) is newly formed along the periphery of xylem in the direction opposite to the rotation, with its one end immediately adjacent to the outside of the old zone and with the other end extending toward the new lower side (Figs. 1, 2, and 3, L_2). It thus occupies half of the girth of xylem for rotation 45°, over half of it for rotation 90°, and for rotation 135° it covers nearly two-thirds of it, extending to a site near the new lower side past the old lower one (Fig. 3B, L_1). As the new zone of mucilaginous layers is thickest in the vicinity of the new upper side (Figs. 1, 2, and 3, U_2), it does not take such a symmetrical form as in the old zone.

Though the new zone of mucilaginous layers varies in thickness according to the duration of the horizontal fixation after rotation, it is commonly thinner than the old zone formed during the time of the initial horizontal fixation if both periods of horizontal fixation, before and after rotation, are nearly equal. As compared with the cells in the zone of mucilaginous layers formed on the upper side of the axis merely horizontally fixed, the cells in the new zone formed after rotation are small, tangentially flat, and thick-walled, and the mucilaginous layers themselves are also remarkably thickened, presenting a strong reaction to zinc chloriodide.

The growth in thickness of the xylem is more vigorous on the new upper side than on the new lower, following the increase in the number of cell layers, as seen in the rotation 45° or 90°. But it is less remarkable in the rotation 135°. This is perhaps due to the facts (i) that the xylem on the new lower side had already attained a considerable thickness while it was situated near the old upper side; and (ii) that the xylem on the new upper side was formed very thinly while it was situated near the old lower side.

Very noticeable in the horizontal axis rotated through 90° is the distribution of starch grains in ray cells (Fig. 2B, *mr*). It is very sparse or scarce in

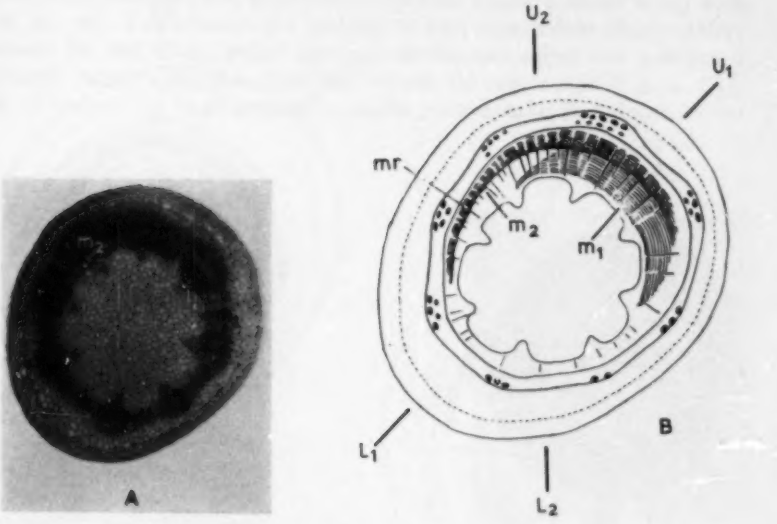

FIG. 1.

FIG. 1, FIG. 2, and FIG. 3. Transection of the horizontal castor-bean hypocotyl rotated through 45°, 90°, and 135°, respectively. *A*, entire section. *B*, drawing interpreting details of *A*. L_1, lower side before rotation. L_2, lower side after rotation. U_1, upper side before rotation. U_2, upper side after rotation. m_1, old zone of mucilaginous layers formed before rotation. m_2, new zone of mucilaginous layers formed after rotation. *mr*, ray tissue (a black line represents a portion filled with starch grains).

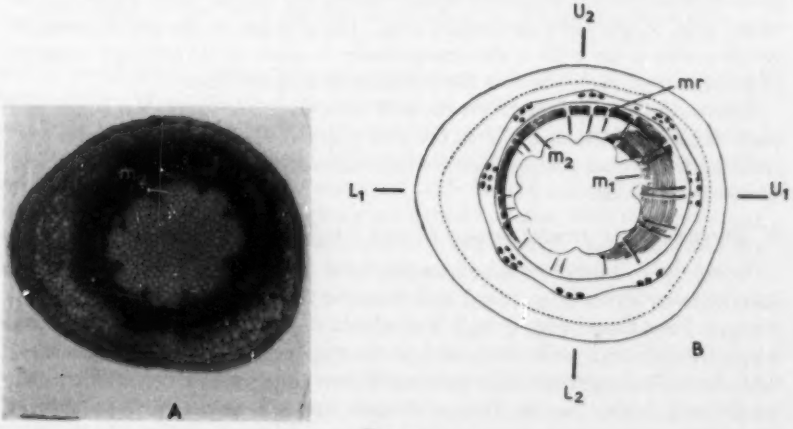

FIG. 2.

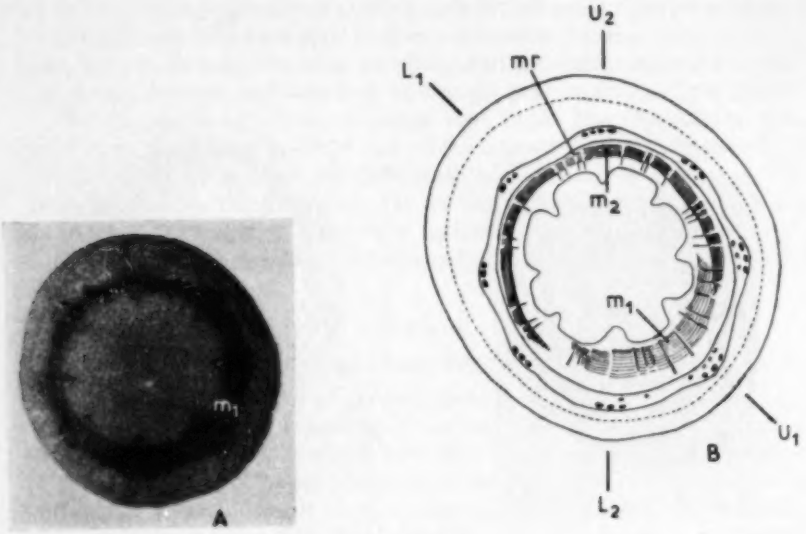

FIG. 3.

almost all the ray cells formed after the rotation, while in the ray cells of the vertical axis it is uniform in density. This is particularly noticeable near the part where both old and new zones of mucilaginous layers overlap each other, or on the new upper side. So far as the distribution of starch grains in the ray cells already formed before rotation is concerned, it is the densest on the new upper side. Nevertheless, the disappearance of starch grains from the ray cells formed after rotation may perhaps be the result of their consumption in the formation of the mucilaginous layers, which are thicker than those on the upper side of the mere horizontal axis. The change in the distribution of starch grains in ray cells is also recognizable in cases of 45° and 135° rotation (Fig. 1B and 3B, *mr*), but it is less remarkable in these cases.

Xylem cells newly formed on the new lower side are somewhat larger and more thick-walled than those on the new upper side. In the case of the 135° rotation, there were some samples which showed tolerably wide intercellular spaces on this side.

B. *Horizontal axis rotated around its own longitudinal axis by 180°*

Xylem cells newly produced on the new upper side (Fig. 4, U_2) form mucilaginous layers (Fig. 4, m_2), and those on the new lower side (Fig. 4, U_1) cease to form them (Fig. 4, m_1). Two zones of mucilaginous layers are thus recognizable facing each other, one on the upper and the other on the lower side. As to the dimensions, the zone on the new upper side is thinner than that on the new lower, but the former extends through almost half the girth of the xylem, occupying a considerably wider area than the latter. On the new

upper side, moreover, the distribution of starch grains is dense in ray cells (Fig. 4B, *mr*). Furthermore the reaction to zinc chloriodide clearly differs between the two sides, in that the zone on the new upper side presents a strikingly darker coloration than that on the old upper side (Fig. 4, U_1); and, of course, the latter presents a similar coloration to that shown in the

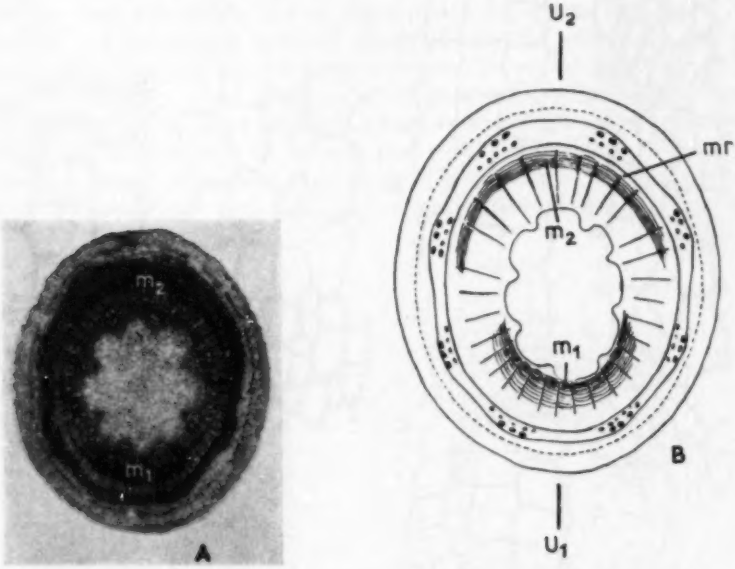

FIG. 4. Transection of the horizontal castor-bean hypocotyl rotated through 180°. U_1, upper side before rotation. U_2, upper side after rotation. m_1, old zone of mucilaginous layers formed before rotation. m_2, new zone of mucilaginous layers after rotation. *mr*, ray tissue (a black line represents a portion filled with starch grains).

zone of mucilaginous layers formed on the upper side of the axis merely fixed horizontally. This difference in intensity of reaction seems to be closely associated with the fact that the new zone of mucilaginous layers is composed of a greater number of layers of cells with heavily lignified, thickened walls and thick mucilaginous layers, compared with the old zone of mucilaginous layers. Moreover, noticeable changes are evident in size, wall-thickness, and enlargement of cells following the rotation. These changes, observable on the radii connecting both upper and lower sides, are here recorded.

First, it is clear that the area with mucilaginous layers on the new lower side (Fig. 5A, *y* and *x*; Fig. 5B, *x'*) is the one differentiated on the old upper side during the time of the initial horizontal fixation. The cells composing its innermost portion (Fig. 5A, *y*) are small, and have considerably thickened walls and mucilaginous layers (Fig. 5A, m_1). The cells successively formed are elongated and enlarged radially, reducing the thickness and lignification of

walls and the thickness of mucilaginous layers one after another (Fig. 5A, *x*; Fig. 5B, *x'*). Thus, the physical rigidity of walls appears to be remarkably lowered. This may be presumed from the fact that the cells are disorderly in

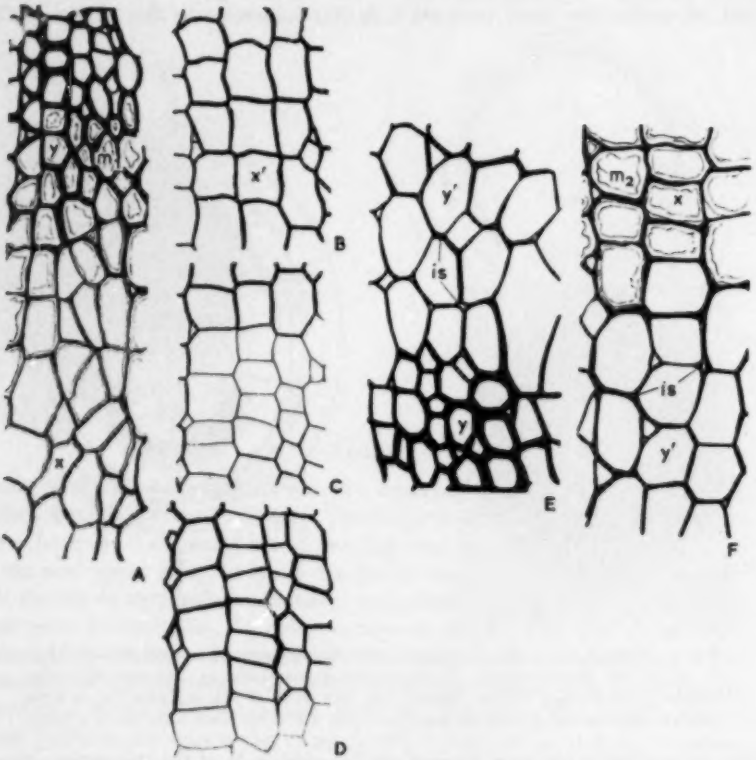

FIG. 5. Diagrams of portions of xylem in the horizontal castor-bean hypocotyl rotated through 180°, showing the changes in tissue structure. *A*, *B*, *C*, and *D*, xylem portions on the lower side after rotation, extending from the inner portion to the outer in order. *E* and *F*, xylem portions on the upper side after rotation, extending from the inner portion to the outer in order. *is*, intercellular space. m_1 and m_2, mucilaginous layers. *x*, *x'*, *y*, and *y'*, xylem cell. (×160)

arrangement, showing irregular curving on their walls, as seen in the portion near the point '*x*' in Fig. 5A. But the cells formed outside the ones mentioned just above (Fig. 5B) recover the regular shape and arrangement characteristic of the normal xylem tissue, and those in the outermost part (Fig. 5C and D) come to increase in the thickness and lignification of walls gradually, ceasing to form mucilaginous layers. From only the fact that these cells do not form mucilaginous layers at all, it may be deduced that they were formed on the new lower side after rotation through 180°. In the xylem on the new upper

side (Fig. 5E and F), the innermost portion is composed of several layers of thick-walled cells (Fig. 5E, *y*) and its outer portion is composed of several layers of radially elongated and thin-walled cells (Fig. 5E and F, *y'*). The portion composed of these large, thin-walled cells is regarded as the one formed in the position of the lower side during the time of the initial horizontal fixation. This may be easily seen from the evidences that the cells in this portion show intercellular spaces (Fig. 5E and F, *is*), and are enlarged, thick-walled, and increasingly lignified, compared with the cells composing the zone of mucilaginous layers formed in the position of the upper side during that time (Fig. 5A, *y* and *x*; Fig. 5B, *x'*). Accordingly, its outer portion must be the one formed in the position of the new upper side after the rotation. However, its cells (Fig. 5F, *x*) are rather small, thick-walled, and heavily lignified, taking the regular arrangement characteristic of the normal xylem and, moreover, they have well-marked mucilaginous layers (Fig. 5F, m_2).

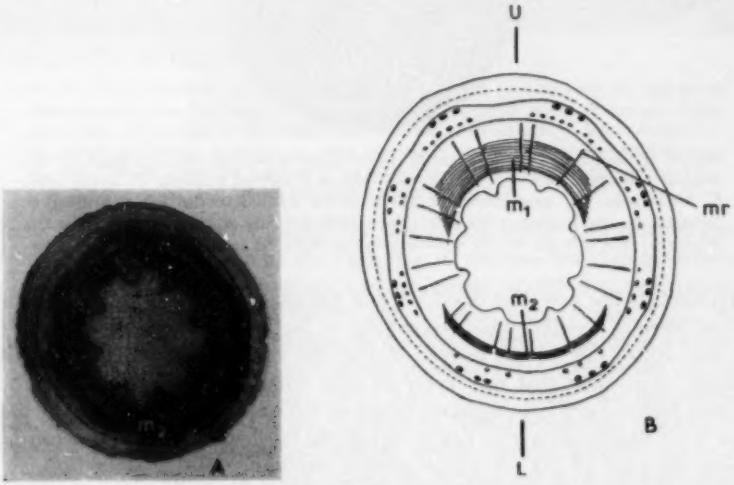

FIG. 6. Transection of the horizontal castor-bean hypocotyl brought to the vertical position by turning through 90°. *L* and *U*, lower and upper sides before turning, respectively. m_1, old zone of mucilaginous layers formed before rotation. m_2, new zone of mucilaginous layers after rotation. *mr*, ray tissue (a black line represents a portion filled with starch grains).

C. *Horizontal axis brought to a vertical position by turning through 90°, and later restored to the horizontal*

If the horizontal axis is turned to a vertical position, xylem cells newly formed on the old upper side (Fig. 6, U) cease from the further formation of mucilaginous layers (Fig. 6A and B, m_1), and those newly formed on the old lower side (Fig. 6, *L*) start the new formation of them (Fig. 6A and B, m_2). But this is only a temporary phenomenon, and cells similar to those in the

xylem of the vertical axis are soon produced. The new zone of mucilaginous layers is thus very thin, being composed of only a few cell layers.

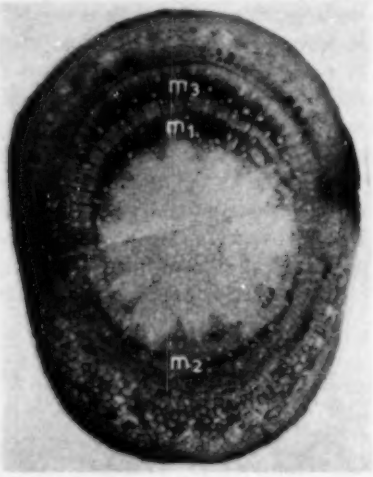

FIG. 7. Transection of the horizontal castor-bean hypocotyl restored again to the original horizontal position, after having been in a vertical position. *U*, upper side. *L*, lower side. m_1, zone of mucilaginous layers formed in the initial horizontal position. m_3, that formed in the second horizontal position. m_2, that formed while the hypocotyl was brought to the vertical position from the initial horizontal position. (×16)

Further, if the axis thus fixed is again restored to the horizontal position, a third zone of mucilaginous layers (Fig. 7, m_3) is formed independently of the first zone (Fig. 7, m_1) formed during the time of the initial horizontal fixation. It is clear that the layers of cells lying between these two zones without forming any mucilaginous layers on the upper side, as well as the thin zone of mucilaginous layers (Fig. 7, m_2) on the lower side, are the ones formed during the time of the preceding vertical fixation.

Generally the cells composing the zone of mucilaginous layers on the upper side of the horizontal axis tend to be radially elongated and thin-walled, reducing lignification. Accordingly, if the transection of the axis kept alternately in a horizontal and vertical position is treated with safranin or with phloroglucin and HCl, a marked change in tissue structure and in degree of lignification in the xylem is apparent in its upper half, as illustrated in Fig. 8A and B.

DISCUSSION

It is already known that a difference in the distribution of some chemical substances may arise between the upper and lower sides of a leaning axis (Priestley and Tong, 1927; Kraus, 1882; Greenwood and Pearsall, 1926; Warner, 1928; Sato, 1952, 1952*a*, 1953). It has also been pointed out that

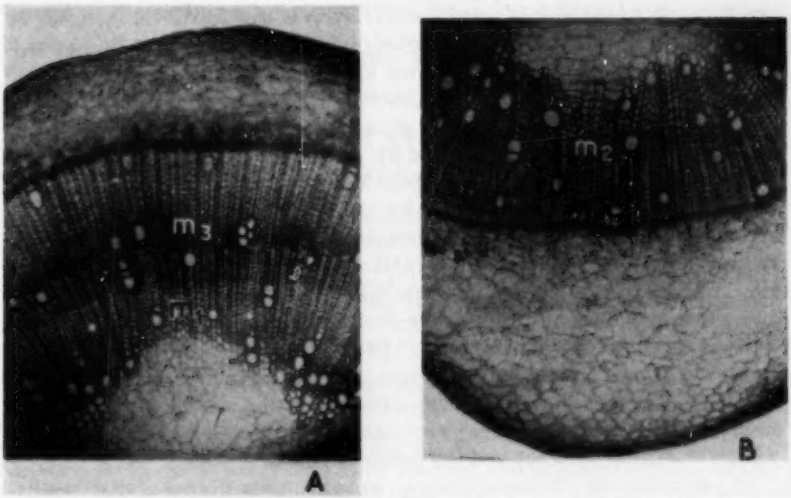

FIG. 8. Transection of the same hypocotyl shown in Fig. 7, showing the change in tissue structure, especially on the upper side (stained with safranin). *A*, a sector of the upper side. *B*, a sector of the lower side. m_1, zone of mucilaginous layers formed in the initial horizontal position. m_2, that formed in the second horizontal position. m_3, that formed while the hypocotyl was brought to the vertical position from the initial horizontal position. (×25)

lateral transport of auxin is caused by exposure to lateral gravitational forces (Dolk, 1929, 1929*a*), and that the formation of reaction wood in a horizontal or a leaning axis is closely associated with a greater accumulation of auxin on the lower side (Wershing and Bailey, 1942; Onaka, 1949; Frazer, 1952; Sato, 1956). Moreover, the disappearance or decrease of starch grains in a swelling which occurred on a plant axis near the point of auxin application has been demonstrated (Borthwick *et al.*, 1937; Sato, 1952, 1952*a*). All the evidence goes to show that in a plant axis exposed to lateral gravitational forces, the substances, such as enzymes and auxins, which regulate both formative and metabolic activities of cells, are caused to be unequal in their distribution, production or activation, &c., between the upper and lower sides.

If such a horizontal axis is rotated around its own longitudinal axis, inequality in the distribution of substances may again occur between the new upper and new lower sides following a gravitational stimulation. Thus, the zone of mucilaginous layers may stop its further formation on the old upper side, and may be newly laid down on the new upper side after rotation. Since protoplasm is endowed with accommodating ability, it ought to accommodate to the unequal distribution of substances brought about on the respective sides of the horizontal axis. The accommodation will remain effectual, though only for a while, and will exert an influence on the formative and metabolic responses to the gravitational stimulus. The greater the angle of rotation, the more powerful the influence of accommodation will be. Both the area and

region where a new zone of mucilaginous layers is laid down will therefore be determined in proportion to the angles rotated. Accordingly, it follows that the new zone of mucilaginous layers is formed asymmetrically in the region extending from the new upper side toward the new lower one in the direction opposite to that of the rotation, occupying a larger area in the horizontal axis rotated by 135° than in that rotated by 45° or 90°. Thus, in the horizontal axis rotated by 180°, the cells just on the new upper side and those just on the new lower side ought to come under the most powerful influence of the accommodation to the preceding horizontal fixation as well as that of gravitational stimulus. It is therefore natural that on the new upper side the new zone of mucilaginous layers is symmetrically formed and that the mucilaginous layers themselves are noticeably more thickened than those formed before the rotation; and that, in the cells newly produced on the new lower side, the formation of mucilaginous layers ceases, and the thickening and lignifying of walls are accelerated. It is similarly reasonable that, in the horizontal axis rotated by 45° to 135°, mucilaginous layers newly formed after rotation are thicker than those already formed before rotation.

In particular, the phenomena shown in xylem after the horizontal axis has been brought to a vertical position are regarded as sufficient evidence to warrant the above consideration. When the respective cells on both upper and lower sides were brought on the same level with each other by turning to a vertical position, their formative and metabolic activities are presumably carried on under the influence of the accommodation to the preceding horizontal fixation, without being affected by the direct influence of gravitational forces. Accordingly, it follows that the xylem cells with mucilaginous layers similar to those formed on the upper side during the time of the initial horizontal fixation are formed on the old lower side, and the xylem cells with thickened and heavily lignified walls similar to those formed on the lower side during the time of the initial horizontal fixation are formed on the old upper side, so long as the postulated accommodation remains effectual.

In short, it may be reasonably concluded that in the horizontal axis rotated around its own longitudinal axis through given angles, the accommodation to the preceding horizontal fixation exerts a powerful influence upon the formative and metabolic responses to gravitational stimulation in proportion to the extent of rotation, and that in the horizontal axis turned to a vertical position the formative and metabolic activities are for a while carried on under the influence of the accommodation alone; for the immediate influence of gravitational forces is eliminated by the treatment.

LITERATURE CITED

BORTHWICK, H. A., HAMNER, K. C., and PARKER, M. W. (1937). Histological and microchemical studies of the reactions of tomato plants to indoleacetic acid. *Bot. Gaz.* **98**, 491.

DOLK, H. E. (1929). Über die Wirkung der Schwerkraft auf Koleoptilen von Avena sativa. *Proc. Kon. Akad. Wetensch. Amsterdam*, **32**, 37.

—— (1929*a*). Über die Wirkung der Schwerkraft auf Koleoptilen von Avena sativa II. Ibid. 1127.

FRAZER, D. E. (1952). Initiation of cambial activity in some forest trees in Ontario. *Ecology*, **33**, 259.

GREENWOOD, D., and PEARSALL, W. H. (1926). Observations on geotropism. *Proc. Leeds Philosoph. Lit. Soc.* **2**, 87.

KRAUS, G. (1882). Über die Wasserverteilung in der Pflanze. *Festschr. d. Naturf. Ges. z. Halle.* **15**, 49.

ONAKA, F. (1949). Studies on compression and tension wood (Japanese, English sum.). *Wood research. Kyoto Uni.* **1**, 1.

PRIESTLEY, J. H., and TONG, D. (1927). The effect of gravity upon cambial activity in trees. *Proc. Leeds Philosoph. Lit. Soc.* **1**, 199.

SATO, I. (1952). Unilateral effect of gravity upon the growth in thickness of *Abelmoschus glutino-textilis* Kag. (Japanese, English sum.). *Mem. Nara Gakugei Uni.* **1**, 187.

—— (1952*a*). Effect of α-naphthaleneacetic acid on the histological structures of the axis of castor-bean plant (Japanese, English sum.). *Proc. Crop Sci. Soc. of Japan*, **22**, 22.

—— (1953). Experimental studies on the effect of gravity upon the tissue formation in the main axis of *Ricinus communis* L. (Japanese, English sum.). Ibid. **2**, 139.

—— (1956). Studies on the georeaction shown in the axes of some herbaceous plants. *Jap. Jour. Bot.* **15**, 249.

—— (in press). Comparative studies on the histological effect of gravity and that of α-naphthaleneacetic acid upon the main axis of *Ricinus communis* L. *Bot. Gaz.*

WARNER, TH. (1928). Über die Einfluss der geotropischen Reizung auf den Zucker-und Säuregehalt von Sprossen. *Jahrb. wiss. Bot.* **68**, 431.

WERSHING, H. F., and BAILEY, I. W. (1942). Seedlings as experimental materials for the study of 'redwood' in conifers. *Jour. For.* **4**, 411.

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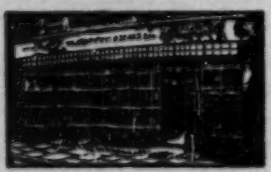

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